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I verify that the attached English translation is a true and correct translation made by me of a certified copy in the German language of European patent application No 00110110.4;

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Date: December 8, 2005

M W R Turner

PCT/EP00/10058

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Certificate

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Patent application No

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Applicants:
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Immunochromatographic rapid test for detecting acid-resistant microorganisms in the stool

The description of this invention mentions a number of published documents. The subject-matter of those documents is incorporated into the description by reference.

The invention relates to an immunochromatographic rapid test, in particular a test strip, for detecting an infection of a mammal with an acid-resistant microorganism. Therein (a) a stool sample of the mammal is incubated with (aa) a receptor under conditions permitting a complex formation of an analyte or an antigen from the acid-resistant microorganism with the receptor; or (ab) at least two different receptors under conditions permitting a complex formation of an analyte or an antigen from the acid-resistant microorganism with the at least two receptors and wherein the receptor according to (aa) or the receptors according to (ab) specifically bind(s) an analyte or an antigen which, at least with some mammals, has a structure after passage through the intestine that corresponds to the native structure or the structure against which a mammal produces antibodies after being infected or immunized with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; and (b) the formation of at least one analyte- or antigen-receptor complex according to (a) is detected. Preferably, the acid-resistant microorganism is a bacterium, in particular *Helicobacter pylori, Helicobacter hepaticus, Campylobacter jejuni* or *Mycobacterium tuberculosis*. Moreover, the receptor(s) preferably bind(s) to (an) epitope(s) of a catalase. Furthermore, the invention relates to diagnostic compositions and test devices containing said components and packages containing the same.

Today, there are various invasive, semi-invasive or non-invasive methods of detecting the infection of a mammal organism with a microbial pathogen or parasite. All invasive methods presuppose endoscopy and biopsy. If these techniques are used, the physical integrity of the

examined subject is violated, e.g. in a biopsy. Obtaining a specimen by biopsy is time-consuming, costly and mostly involves a high strain on the patient. As the infection with particular microorganisms, for instance with *H. pylori*, need not be distributed over the entire gastric mucosa, obtaining a specimen by biopsy at a non-infected site may deliver a false-negative result. Another disadvantage of all invasive methods is that the examination results are influenced by earlier treatment with proton-pump inhibitors, bismuth or antibodies.

Semi-invasive or non-invasive diagnostic methods note changes in parameters which may be measured without interfering in the organism. For this purpose preferably samples of body fluids and excretions, such as serum, breath, urine, saliva, sweat or stool are taken and analysed.

Diagnostic techniques can be divided into direct or indirect methods on the basis of the detected parameters. With direct methods the presence of the pathogen or parasite, its components or their degradation products is detected by electron microscopy, optical characterisation, mass spectrometry, measurement of the radioactive degradation products or specific enzymatic reactions. However, these methods often require expensive and sophisticated instruments (e.g. the breath test). By contrast, indirect methods are used for detecting reactions of the host organism to the pathogen or the parasite, for instance the presence of antibodies against antigens of the pathogen in the serum or the saliva of the host.

Since interfering in the organism using invasive techniques strains the organism in most cases and frequently also requires expensive and sophisticated instruments and involves a health hazard, non-invasive techniques are the methods of choice since it is comparatively simple to take samples of the above-mentioned body fluids and excretions. Furthermore, since not every host reacts in the same way to a given pathogen or parasite, and the host reaction may be delayed and also may

persist even after the pathogen or parasite has been removed from the organism, direct methods are always to be preferred. Hence, ideally, a diagnosis is made by means of the non-invasive, direct detection of the pathogen or parasite in body fluids or excretions. Contrary to indirect methods, this allows the current infection status to be determined.

Moreover, a diagnostic method should also be optimised with regard to other aspects: high reproducibility, sensitivity and specificity, guaranteed availability and constant quality of the materials to be used, low costs in production and carrying out the method and simple application independent of expensive and sophisticated instruments are the parameters to be taken into consideration here.

For the above-mentioned reasons, in medical diagnostics increasing use is being made of methods based on the high selectivity and binding affinity of given classes of substances (e.g. antibodies, receptors, lectins and aptamers) for molecular structures which can be selected in such a way that they are highly specific for the corresponding substance to be analysed. It was in particular the possibility of immobilising those substances on surfaces of solids as well as coupling to radioactive nuclides, enzymes triggering colour reactions with suitable substrates, or coloured particles with a highly specific binding affinity (e.g. ELISA = enzyme-linked immunosorbent assay) that led to the development of inexpensive, simple and less time-consuming methods of detecting substances that are naturally occurring in the body or foreign to the body.

In the initial phases of the development of those detection methods exclusively polyclonal antibodies were used. They have some disadvantages well known to the man skilled in the art however, thus mainly limited availability and often also cross-reactivity. The development of methods of preparing monoclonal antibodies (Köhler & Milstein (1975)), the advances in the isolation of receptors and their directed expression in cellular host systems, the development of lectins with high affinity to given

carbohydrates and the discovery that single-stranded nucleic acid molecules (aptamers) are able to specifically bind molecular structures, allowed the majority of those disadvantages to be eliminated. Today, the specificity and sensitivity of detection methods can be optimised with comparatively simple methods.

Due to the high specificity, such methods are particularly suitable for detecting individual defined substances such as haptens, peptides or proteins, provided the structural element that has been recognised is constant within the specimen population to be examined and specific to the substance to be detected. Moreover they are well suited for measurements in body fluids and thus are an obvious option for the direct detection of pathogens in that specimen matrix. Accordingly, the prior art describes methods of diagnosing e.g. *Entamoeba histolytica* (Hague (1993), J. Infect. Dis. 167: 247-9), enterohemorrhagic *Escherichia coli* (EHEC, Park (1996), J. Clin. Microbiol. 34: 988-990), *Vibrio cholerae* (Hasan (1954), FEMS Microbiol. Lett. 120: 143-148), Toro virus-like particles (Koopmans (1993) J. Clin Microbiol. 31: 2738-2744) or *Taenia saginata* (Machnicka (1996), Appl. Parasitol. 37: 106-110) from stool.

The feature that the above-described pathogens have in common is that they are viable and reproducible in the intestine of the host, in all cases humans. Hence they have mechanisms allowing them to survive and propagate in the presence of the degradation and digestion systems active in the intestine. Thus it is probable that a large number of intact or almost intact pathogens or parasites are passed upon excretion with the stool. As a rule, it is easy to detect them in the stool or in prepared stool samples by means of detection reagents, for instance antibodies that recognise the intact pathogens or parasites.

There is however a number of pathogens or parasites that on the one hand may be present in the stool due to the relations of the affected tissue (e.g. lungs, stomach, pancreas, duodenum, liver) to the gastrointestinal

tract and that on the other hand are however not viable and/or reproducible in the intestine itself. These pathogens and parasites include, for instance, *Helicobacter pylori (H. pylori)* and *Helicobacter hepatis, Mycobacterium tuberculosis* and other mycobacteria, *Chlamydia pneumoniae, Legionella pneumophilae, Pneumocystis carinii* and others. Other pathogens such as *Legionella pneumophilae* can be detected specifically by means of antigens which pass into the urine via the kidneys. That however is only possible if the amount in the urine is sufficient for detection. Detection in the stool would be a welcome alternative to that. In these organisms however passage through the intestine is combined with a strong attack by the digestion and degradation mechanisms of the intestinal flora. In this case, molecular structures which are specific to the pathogen observed can be destroyed or their concentration can be greatly reduced.

With other acid-resistant bacteria too degradation of the pathogens in the intestine has turned out to be a problem for reliable detection in stool samples. The number of germs in the stomach of an infected patient is small compared to other bacteria settling in the intestine. Furthermore, germs and germ fragments have to pass a long way through the intestine which is rich in proteases after leaving the stomach. Those circumstances mean that only small amounts of intact proteins can be found in the stool, in which respect however it cannot be assumed that it is always the same fragments of specific proteins that pass through the intestinal tract undamaged. This also means that the combination of two epitopes on one antigen, which is necessary for an ELISA test or immunochromatic rapid test, is no longer necessarily like the one occurring in the native protein and epitopes located close to each other are most likely to show a positive result in a detection method requiring two epitopes on the same molecule. Ideally, only one epitope on the same molecule is needed for detection, wherein that epitope, in the case of a monomer, must be present twice

thereon. In the case for example of a dimer a single presence on each subunit would suffice. In addition the individually different distribution of antigens detected in the stool of infected patients suggests individual features in processing of the antigens during passage through the intestine. A first approach to reducing this problem was provided by the disclosure of EP-A 0 806 667. In that application it was shown that polyclonal antibodies could be induced with the lysate of a given *H. pylori* strain, which recognise a greater variability of strains from different geographical regions. However this application does not indicate which antigens are recognised by the serum. In view of the fact that immune sera may vary in spite of all standardisation efforts, the method developed in the above-mentioned application must be regarded as suboptimal for broad application. In addition, it is necessary to repeatedly immunise new animals in order to provide the polyclonal sera. The corresponding methods are both time-consuming and costly.

Ideally reliable detection of the infection of an acid-resistant pathogenic organism/parasite as mentioned above is possible with a single or a limited number of reagent(s) specific to that pathogenic organism/parasite.

EP 291 194 describes an analytical test apparatus which includes a porous support with a specific binding reagent, which is mobile in the moist state, for an analyte and a permanently immobilised, unlabelled specific binding reagent for the same analyte.

Hence, the underlying problem of the present invention is to provide a corresponding simple and inexpensive test.

That problem is solved by the embodiments characterised in the claims.

The invention relates to a method of detecting an infection of a mammal with an acid-resistant microorganism, wherein (a) a stool sample of the mammal is incubated with (aa) a receptor under conditions

permitting a complex formation of an antigen from the acid-resistant microorganism with the receptor; or (ab) at least two different receptors under conditions permitting a complex formation of an antigen from the acid-resistant microorganism with the at least two receptors, and wherein the receptor according to (aa) or the receptors according to (ab) specifically bind(s) an antigen which at least in some of the mammals has a structure after passage through the intestine that corresponds to the native structure or the structure against which a mammal produces antibodies after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; and (b) the formation of at least one antigen-receptor complex according to (a) is detected.

The invention is further based on the idea of providing an immunochromatographic rapid test such as a test strip which is suitable for providing for detection of a specified infection.

The stool rapid test includes a plurality of locations or regions which preferably comprise possibly different porous materials. The test strip has a sample application region, the actual test support (test or analysis region) and an absorber layer (absorption region). In a preferred embodiment the plurality of locations or regions are fixedly arranged on a polyester support. In a preferred embodiment, in the dried condition, disposed in the sample application region are the specific immunological receptors required for the detection procedure and further preferably specific antibodies for the antigen. They are preferably labelled with particles which are coloured so as to visible, for example colloidal gold or latex or polystyrene etc.

The test support further preferably comprises a specific test membrane such as for example nitrocellulose. Particularly preferably further specific receptors which are directed against the antigen are immobilised on that test membrane as a test line. As a function control a further catching line can be immobilised on the test membrane, for

example antibodies or receptors directed against the labelled antibody or receptor. An absorber layer at the end of the test strip advantageously provides that the sample flow which is based on the capillary action of the porous materials which are in contact with each other is maintained.

In the method according to the invention of sandwich immunoassay, in an embodiment, a labelled first specific receptor for the analyte or the antigen, for example a labelled antibody (antibody conjugate), is deposited or dried on in the sample application region of the test strip. A second specific receptor for the analyte or the antigen is immobilised as a test line. During the test the first specific receptor such as for example an antibody conjugate is dissolved by the sample liquid and transported over the test membrane. If the specific analyte or the antigen is present in the sample complexes of labelled first receptor or antibody conjugate and antigen or analyte are formed during the test run. At the catching line that complex binds to the second specific receptor and there forms the so-called sandwich complex. A visible test signal is produced due to the accumulation, related thereto, of the labelled receptors or antibody conjugates at the test line. If no analyte or antigen is present in the sample no sandwich complex is formed and no signalling is implemented.

In a preferred embodiment of the sandwich method streptavidin is immobilised to the test line instead of a specific receptor. The specific receptor for the antigen, which is used in the simple sandwich method as a catcher on the test line, is conjugated to biotin and deposited together with a labelled specific receptor in the sample application region of the test strip. In a particularly preferred embodiment the labelling is colloidal gold. During implementation of the test a sandwich complex comprising a labelled receptor, the antigen and the biotin-labelled receptor is already formed during the migration through the sample application region and the test membrane and is bound at the test line by way of the biotin-labelled specific receptor by the streptavidin immobilised there.

In a further preferred embodiment the gold-labelled specific receptor is deposited in a first conjugate region of the sample application region of the test strip and the biotin-labelled specific receptor is deposited in a second conjugate region of the sample application region.

In a further preferred embodiment the labelled first specific receptor can be replaced by a non-labelled first specific receptor, for example antibody. That first specific receptor is then detected by a further labelled receptor which binds that first specific receptor, in which case the further labelled receptor does not bind the second specific receptor which is immobilised as the test line.

In a further preferred embodiment the specific second receptor immobilised on the test line can be replaced by a non-immobilised second specific receptor, for example an antibody. The analyte-receptor complex is then bound by a receptor which is immobilised on the test line and which binds that non-immobilised second specific receptor, in which case the further immobilised receptor does not bind the first labelled specific receptor.

In a particularly preferred embodiment the non-immobilised second specific receptor is present bound to the receptor immobilised on the test line.

When using antibodies that can be implemented for example by the first and second specific antibodies originating from different species.

By way of example the first specific non-labelled receptor is a mouse-antibody, the second specific receptor is a rabbit-antibody and the further labelled receptor is an anti-mouse-antibody.

In a particularly preferred embodiment the specific non-labelled receptor is deposited in a first conjugate region of the sample application region of the test strip and the marked receptor which binds the specific non-labelled receptor is deposited in a second conjugate region of the sample application region of the test strip.

In a further particularly preferred embodiment the first conjugate region is arranged in the sample application region in front of or over the second conjugate region in the flow direction.

An immunochromatographic rapid test is particularly suitable for implementation of the present invention. That test is a dry reagent test, wherein all specific reagents necessary for the analysis operation are contained in a test strip composed of a plurality of porous materials, preferably in the dry condition. A test of that kind is based on the principle that the analysis is started by the addition of a liquid sample and the sample liquid passes by virtue of capillary forces through a test strip composed of a plurality of porous materials. During the migration movement of the sample liquid specific binding reagents are dissolved and complex formation occurs between analyte contained in the sample and specific binding reagents. The complexes of analyte and specific binding reagents are caught at a defined zone which is preferably in the form of a test line by a specific binding reagent which is immobilised at that test location. Those caught complexes are rendered visible by the accumulation of the visible particles coupled to the binding reagents, for example coloured polystyrene, colloidal gold and so forth. In that way the test according to the invention can also be carried out by non-specialists. In addition it permits simple and hygienic handling. In carrying out the operation, in the particularly preferred embodiments, only one step is required, namely application of a sample. The visually evaluatable result is then available very quickly within a few minutes (2 – 30 minutes).

In another embodiment the immunochromatographic rapid test according to the invention is used in the test apparatus described in WO 98/58587. That combination permits rapid and easy sample collection, preparation and analysis.

In addition the rapid test according to the invention avoids the disadvantage encountered hitherto in the analysis of stool samples, namely

that the sample dissolved in the buffer has a high content of solids which cause difficulties in regard to or prevent the flow of sample through the fine porous materials of the test strip.

In addition the disadvantages in the state of the art can be avoided, namely that the stool samples have to be greatly diluted. In addition when carrying out the method according to the invention the stool samples which are diluted in the range of 1:5 to 1:20 do not have to be centrifuged and freed of relatively large solid constituents prior to the test. In addition the present invention makes it possible to provide a highly sensitive test for laboratory-independent use.

In particular the displacement of the preferred biotinylated receptor from the test line into the application region provides for a greatly prolonged incubation time for binding of the catcher antibody (second receptor) to the antigen (analyte) by virtue of the higher level of affinity of the streptavidin/biotin binding in relation to the antibody/antigen binding and thus better binding kinetics at the test line although the test strip can remain almost unchanged in respect of its dimensions.

In accordance with the present invention, the term "acid-resistant microorganism" encompasses any microorganism which, due to its properties/mechanisms of adapting to the host, withstands the physical and chemical influences of the digestive tract so that it can be detected by a preferably immunological test or by the use of aptamers. Examples of such acid-resistant microorganisms are *Helicobacter pylori, Helicobacter hepaticum, Mycobacterium tuberculosis, Mycobacterium pseudotuberculosis* and *Mycobacterium cansassii*.

The term "stool sample of the mammal" in accordance with the present invention means any stool sample which can be used for the detection method of the invention. In particular it includes stool samples which have been prepared for diagnostic tests according to per se known methods. Preparation is carried out for instance according to

RIDASCREEN® Entamoeba enzyme immunoassay (R-Biopharm GmbH, Darmstadt).

The man skilled in the art can readily adjust "conditions permitting complex formation", cf. also Harlow and Lane, ibid. These conditions are for example physiological conditions.

The term "has a structure after passage through the intestine that corresponds to the native structure", in accordance with the present invention, means that the epitope of an antigen is recognised after passage through the intestine by a receptor, e.g. a monoclonal antibody, derivative or fragment thereof or the aptamer which was obtained against the same antigen/epitope that has not passed the intestine or which binds thereto. In other words, the epitope/antigen that is specifically bound by the above has passed the intestine intact or essentially intact as regards its structure and has not been degraded. A source for the native structure of the epitope/antigen may be for instance a bacterial extract that was disrupted by means of a French press and further purified with standard methods (cf., for instance, Sambrook et al., "Molecular Cloning, A Laboratory Manual", 2nd edition, 1989, CSH Press, Cold Spring Harbor USA) or a bacterial lysate further purified according to standard methods (e.g. Sambrook et al., ibid.).

According to the invention, the term "has a structure after passage through the intestine that corresponds to the structure against which a mammal produces antibodies after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide" means that the epitope recognised by the receptor corresponds to an epitope which is presented by the immune system of a mammal, preferably a human. The mechanisms of antigen presentation as well as the mechanisms leading to the processing of antigens and the variety of antibodies resulting therefrom are known in the prior art and are described for instance in Janeway and

Travers, Immunologie, 2nd edition 1997, Spektrum Akademischer Verlage GmbH, Heidelberg. Those epitopes may differ from native epitopes. Contact of the mammal with the microorganisms or proteins or fragments or the synthetic peptides can be brought about by natural infection (except for synthetic peptides) or by immunisation. For immunisation extracts, lysates, synthetic peptides, etc. of the microorganism/protein can also be used. Suitable immunisation methods are known in the prior art and are described for instance in Harlow and Lane, ibid. Suitable antibodies may also be obtained for example by immunisation and/or screening for surrogates such as synthetic peptides, recombinantly produced proteins, extracts, lysates or partially digested proteins.

"Synthetic peptides" comprise those peptides having at least one epitope of the native antigen or the antigen which has passed through the intestine. The peptides can have the same primary structure as the antigen or fragments thereof. However they can also have a different primary structure (primary amino acid sequence, for instance conservative exchanges).

The terms "specifically binds" means according to the invention that the receptor shows no or essentially no reactivity with other epitopes in samples of non-infected mammals.

The term "immune complex" according to the invention includes complexes, including monoclonal and/or polyclonal antibodies.

In this embodiment of the invention a prepared stool sample can be bound for instance by way of a catching receptor to a solid phase and the infecting agent can be detected with the labelled receptor. If the antigen which is present after having passed the intestine is (still) present in (homo-)dimeric or multimeric form, the same receptor can be used both as a catcher and as a detector.

In addition, it is of importance for the method of the invention that successful detection requires only one epitope of an antigen protein to be

detectable after passage through the intestine in an essentially consistent manner. That epitope can also occur several times on a homo-dimer or –multimer. The likelihood of finding that epitope in detectable form is however significantly higher than if a detection test has to rely on more than one epitope to be detected.

Finally the method of the invention requiring only one receptor involves advantages as regards costs and standardisation.

On the basis of the surprising finding according to the invention that given antigens from said microorganisms have an epitope structure after passage through the intestine which can be essentially consistently detected a second embodiment must also be considered essential to the invention. This embodiment is based on the fact that different receptors bind to different epitopes of the same antigen. Here the term "essentially" means that the epitopes and thus a corresponding infection with the microorganism can be detected in more than 60 to 70%, preferably at least 75%, more preferably more than 85%, particularly preferred more than 90%, even more preferably more than 95% and most preferably more than 98% of the infected individuals. Ideally infections are detected in 100% of the infected individuals.

According to the invention it was surprisingly found that by means of a single receptor which specifically binds an epitope of an antigen of an acid-resistant microorganism, or two receptors which specifically bind two epitopes of the same antigen infection with those bacteria/pathogens can be relatively reliably diagnosed. The invention includes embodiments in which other epitopes having said properties are recognised by other receptors, for instance by monoclonal antibodies or fragments or derivatives thereof or aptamers. The latter embodiments are suitable for further increasing the reliability of the diagnosis. Advantageously those other receptors may be antibodies, fragments or derivatives, which specifically recognise urease, preferably ß-urease, the 26 kDa protein or

Hsp 60, all preferably from *H. pylori*. The detection of one or more of those proteins/protein fragments may be carried out in the same test or in an independent test with another part of the same sample.

The results of the invention are surprising mainly because the state of the art had taught away therefrom. In the case of *H. pylori* for example it was found that main antigens do not show the desired specificity and sensitivity in ELISA tests; cf. Newell et al., Serodiag. Immunother. Infect. Dis. 3 (1989), 1-6. Moreover, EP-A-0 806 667 teaches that it is not possible to reliably detect *H. pylori* infections with receptors such as monoclonal antibodies due to the genetic variability of the *H. pylori* strains.

Compared to the aforementioned state of the art the method of the invention is of advantage in particular since it permits a relatively reliable diagnosis with only one receptor. In ELISA or the rapid test for instance pairs of receptors such as antibodies, fragments, derivatives thereof or aptamers are preferably used for detection, with the two receptors of the pair binding the same or different epitopes on the same antigen. *H. pylori* catalase for example forms multimeric structures of several identical subunits. Therefore in ELISA, the rapid test or other assays the same receptors can be used both as catching receptors and also as detection receptors.

Preferably an increase in sensitivity and specificity can be achieved by a combination of different mabs which are directed against different epitopes of the catalase or by a combination of two detection systems for different antigens (for example catalase/urease).

Another advantage of the method of the invention is the fact that it is a direct and non-invasive method, which increases the above-mentioned advantages for the patient and reliability in determining the stage of the disease.

Preferred embodiments of the invention are described hereinafter by way of example with reference to the Figures in which:

Figure 1 shows a cloned DNA sequence coding for the V-region of the heavy chain of a monoclonal antibody specific to catalase (HP25.2m/2H10).

Figure 2 shows a cloned DNA sequence coding for the V-region of the light chain of a monoclonal antibody specific to catalase (HP25.2m/2H10).

Figure 3 shows a cloned DNA sequence coding for the V-region of the light chain of a monoclonal antibody specific to catalase (HP25.2m/2H10).

Figure 4 shows a cloned DNA sequence coding for the V-region of the light chain of a monoclonal antibody specific to catalase (HP25.6m/1B5).

Figure 5 shows a structure of a preferred test strip according to the invention.

Figure 6 shows a structure of a preferred test strip according to the invention with control line.

The stool rapid test according to the invention as shown in Figure 6 comprises a sample application region 1, 2, a test region (test membrane) 3 and an absorption region 4.

In a preferred embodiment the sample application region 1 comprises two mutually superposed conjugate regions. Preferably the upper conjugate region or the conjugate fleece contains the specific receptor labelled for example with gold. The subjacent conjugate region or the conjugate fleece (not shown) contains for example the biotin-labelled specific receptor.

A good sample flow and uniform distribution of the immunoreagents in the sample suspension during the test procedure are of great significance in terms of the sensitivity of an immunochromatographic rapid test. Both parameters are influenced in particular by the properties of the porous materials used as well as their dimensions and mutual arrangement.

In the case of rapid tests for the investigation of stool samples there is additionally the requirement that the solids contained in the sample solution are efficiently filtered off before passing over onto the test membrane.

The sample application region of the test is composed of a conjugate fleece 1 and an adjoining filter 2. The solid constituents of the stool suspension which would prevent a flow of sample over the test membrane are separated off by the filter. The test membranes with which a high level of sensitivity is to be achieved involve a distribution of pore sizes in the range of between 2 and 20 µm, preferably 5 µm. The filter has an exclusion size of 1 – 2 µm. Materials of glass fibre or polyester-glass fibre mixes are particularly suitable for that purpose. Mixes of natural and synthetic fibres which were developed for blood separation are also suitable.

The following filters are for example suitable: Whatman GF/A, GF/B, GF/C, GF/D, F145, F147, F487, GF/DVA; Ahlstrom 111, 141, 142, 151, 164; Pall A/B, A/C, A/D, A/E, A/F. Open-pore material comprising glass fibre, polyester or polypropylene is suitable for the conjugate fleece such as for example Whatman F 075-14; Scheicher und Schuell GF 10, GF33, Ahlstrom 8980, 2033, 2040, 8975; Millipore QR 01, QR 35, QR 51, QR 61.

In that structure, besides its function as a conjugate support layer, the conjugate fleece serves as a pre-filter which retains coarse solid constituents of the suspended stool sample before they encounter the filter. A further advantage with that structure is that sample liquid and dissolved conjugate flow jointly through the downstream-connected filter and thus the incubation time of the analyte or antigen and conjugate is prolonged.

The dimensions of the conjugate fleece, filter and test membrane and the transitions between the materials of the sample application region are such that adequate filtering of the stool suspension is achieved.

During sample application the sample liquid may only come into contact with the conjugate fleece but not with the filter.

The structure according to the invention guarantees that an adequate filter area can come into contact with the sample liquid flowing through the conjugate fleece. In addition the structure according to the

invention permits a minimum spacing between the conjugate fleece and the test membrane in order to exclude sample liquid which has not been adequately filtered from flowing over onto the test membrane.

The dimensions of the individual regions or materials correspond to the following:

The preferred width of the test strip is between 3 mm and 10 mm, particularly preferably 5 mm. The preferred length of the test strip is 50 – 100 mm, particularly preferably 75 mm. The preferred length of the conjugate fleece is in the range of 5 – 30 mm, particularly preferably being 25 mm. In this case the overlap of the conjugate fleece and the filter is preferably between 5 and 15 mm, preferably being 10 mm. Filters of a length of between 10 and 20 mm, particularly preferably being 15 mm, are preferred. The overlap between the filter and the test membrane is preferably between 1 and 3 mm, particularly preferably being 1 mm.

According to the invention the absorber layer at the end of the test strip 4 has both an adequate capacity for absorption of the sample liquid which has passed through the test strip and also a relatively fine-pore structure for maintaining the capillary action. Cellulose-glass fibre materials are found to be particularly suitable for that purpose, such as for example Whatman 17 CHR, 3 MM, 31 ET, WF1.5, D28, CD 427-05, CD 427-07, CD 427-08; Scheicher & Schuell 2992, GB 003, GB 004; Pall 111, 113, 133, 165, 197; Ahlstrom 320, 222, 238, 237. With test strips of a width of 5 mm a dimension of 10 – 30 mm length for the analysis region is particularly preferred. The overlap of the test membrane onto the absorber layer is preferably at least 1 mm.

Particularly preferably the rapid test of the invention is used in an apparatus for taking and investigating samples as is described in WO 98/58587 (PCT/EP98/03764). The subject-matter of that publication is incorporated into this description by reference.

In a preferred embodiment the acid-resistant microorganism is an acid-resistant bacterium.

A number of acid-resistant bacteria are known in the state of the art. In a particularly preferred embodiment the acid-resistant bacterium is a bacterium of the genus Helicobacter, Campylobacter or the genus Mycobacterium.

In another particularly preferred embodiment the bacterium is a bacterium of the species *Helicobacter pylori, Helicobacter hepaticum, Campylobacter jejuni* or a bacterium of the species *Mycobacterium tuberculosis.*

In another preferred embodiment the receptor(s) is/are (an) antibody (antibodies), (a) fragment(s) or (a) derivative(s) thereof or (an) aptamer(s).

In accordance with the present invention however the term "receptor" also includes further binding partners such as for example avidin, streptavidin or polystreptavidin and biotin.

In accordance with the present invention, "fragments" or "derivatives" of monoclonal antibodies have the same binding specificity as the monoclonal antibodies. Such fragments or derivatives can be produced according to usual methods; cf. for example Harlow and Lane, "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, USA, 1988. Examples of fragments include Fab-, $F(ab')_2$ or Fv-fragments. ScFv-fragments are examples of derivatives. Derivatives can also be chemically produced substances having the same binding properties as the antibodies or improved binding properties. Such substances can be produced for instance by peptidomimetics or by different cycles of phage display and subsequent selection to improved binding properties. According to the invention, aptamers are nucleic acids such as RNA, ssDNA (ss=single stranded), modified RNA or modified ssDNA, which bind a large number of target sequences having high specificity and affinity. The term "aptamer"

is known and defined in the state of the art, for example in Osborne et al., Curr. Opin. Chem. Biol. 1 (1997), 5-9 or in Stull and Szoka, Pharm. Res. 12 (1995), 465-483.

The term "antigen-antibody complex" in accordance with the present invention includes not only complexes which the antigen forms with the native antibody, but also those which it forms with the fragments or derivatives thereof.

The invention includes embodiments in which only monoclonal antibodies or fragments or derivatives thereof or only aptamers are used as well as embodiments in which different types of detection reagents are used in one test. Hence it is possible for a first monoclonal antibody to be used with a second antibody derivative or a first aptamer to be used with a second antibody fragment, to name only two examples. In this respect, the terms "first" and "second" refer to the first and the second detection reagent. That however does not mean that two antibodies, derivatives or fragments thereof or two aptamers are always used.

The use of monoclonal antibodies, fragments or derivatives thereof or of aptamers ensures a standard which is to be easily observed in reliability of the diagnosis method, which means a great advantage compared to diagnosis methods that have been known hitherto and that have been introduced for this purpose. Moreover it is no longer necessary to keep re-immunising and subsequently testing test animals as is required for instance in the method according to EP-A 0 806 667.

In another preferred embodiment the antigen is the antigen of a catalase. The catalase has the special advantage that it could be detected in all acid-resistant bacteria known hitherto. According to the invention it was found, as another advantage, that the catalase is highly resistant to digestion in the intestinal tract, which simplifies detection of significant amounts. Finally the catalase or fragments thereof is/are often still present in a superior structure, for instance in tetrameric form, after having passed

through the intestine, which facilitates detection with one receptor type only.

According to the invention, it was surprisingly found that in a population of mammals, in particular human patients, whose stool had been tested for infections with acid-resistant bacteria, essentially all members of this population showed consistently recurring catalase epitopes in the stool, so that a relatively reliable diagnosis can be made with a high degree of probability with only one corresponding receptor, preferably monoclonal antibodies, fragments or derivatives thereof or aptamers. In particular, since the catalase has a tetrameric antigen structure, this diagnosis can advantageously be made for instance in ELISA or in similarly arranged solid systems.

It is particularly preferred for the catalase to be the catalase of *H. pylori*.

In another preferred embodiment additional use is made of a mixture of receptors for detection, with the mixture of receptors having the function of a catcher of the antigen if the receptor is used as detector of the antigen, and the mixture having the function of a detector of the antigen if the receptor is used as catcher of the antigen. For the detection operation it is possible to use both different mixtures of receptors as the catcher and detector of the antigen and also the same mixture as the catcher and detector of the antigen. When using the same mixture as the catcher and detector of the antigen the catcher is preferably in labelled form and the detector immobilised on the test line.

This embodiment of the invention permits particularly reliable diagnosis, particularly if the antigen is not present in a dimeric or multimeric conformation after passing through the intestine. This embodiment makes it possible for only one of the two receptor types used in the majority of standardized immunological detection methods to be a

monoclonal antibody while for instance the second receptor type may be a polyclonal serum.

In a particularly preferred embodiment the mixture of receptors is a polyclonal antiserum.

In a further particularly preferred embodiment mixtures of receptors are used for the detection operation, wherein a mixture of receptors functions as a catcher of the antigen and a mixture of receptors functions as a detector of the antigen and preferably at least one mixture is a polyclonal serum.

In another particularly preferred embodiment a mixture of receptors functions both as catcher and also as detector of the antigen, wherein preferably that mixture is a polyclonal antiserum.

In an additionally particularly preferred embodiment the polyclonal antiserum against a lysate of the microorganism, preferably *H. pylori*, was obtained.

In another particularly preferred embodiment the lysate is a lysate with an enriched antigen.

In another preferred embodiment, the lysate is a lysate with depleted immunodominant antigen.

The two aforementioned embodiments also include the fact that the lysate is a lysate with enriched antigen, preferably with enriched catalase and with depleted immunodominant antigen, preferably mainly antigenic urease. In particular that combination offers the possibility of obtaining a high immunization yield which is especially suitable for the method of the invention. A way of carrying out corresponding enrichment and depletion methods is described in greater detail in the Examples.

The polyclonal antiserum against a purified or a (semi)synthetically produced antigen was obtained according to another particularly preferred embodiment.

According to the invention, the receptors, preferably the monoclonal antibodies, fragments or derivatives thereof or the aptamers, can recognise and specifically bind linear or conformation epitopes. In another preferred embodiment, at least one of the receptors binds a conformation epitope.

In a particularly preferred embodiment, all receptors bind conformation epitopes.

In a particularly preferred embodiment, the heavy chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: NYWIH

CDR2: YINPATGSTSYNQDFQD

CDR3: EGYDGFDS

In another particularly preferred embodiment, the DNA sequence coding the heavy chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: AACTACTGGATTCAC

CDR2: TACATTAATC CTGCCACTGG TTCCACTTCT TACAATCAGG ACTTTCAGGA C

CDR3: GAGGGGTACG ACGGGTTTGA CTCC

In another particularly preferred embodiment, the light chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: SASSSVNYMY

CDR2: DTSKLAS

CDR3: QQWSSNPYT

Furthermore, in another particularly preferred embodiment, the DNA sequence coding the light chain of this antibody [HP25.2m/2H10] has at

least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: AGTGCCAGCT CAAGTGTAAA TTACATGTAC

CDR2: GACACATCCA AATTGGCTTC T

CDR3: CAGCAGTGGA GTAGTAATCC GTACACG

In a particularly preferred embodiment, the heavy chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: DTYVH

CDR2: KIDPANGKTKYDPIFQA

CDR3: PIYYASSWFAY

In another particularly preferred embodiment, the DNA sequence coding the heavy chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: GACACCTATGTGCAC

CDR2: AAGATTGATCCTGCGAATGGTAAAACTAAATATGACCCGATA TTCCAGGCC

CDR3: CCCATTTATTACGCTAGTTCCTGGTTTGCTTAC

In another particularly preferred embodiment, the light chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: KASQDVGTSVA

CDR2: WTSTRHT

CDR3: QQYSSSPT

Furthermore, in another particularly preferred embodiment, the DNA sequence coding the light chain of this antibody [HP25.6m/1B5] has at

least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: AAGGCCAGTCAGGATGTGGGTACTTCTGTTGCC

CDR2: TGGACATCCACCCGGCACACT

CDR3: CAGCAATATAGCAGCTCTCCCACG

In addition, it is particularly preferred that the heavy and light chains having said CDRs occur together in one antibody, fragment or derivative thereof, which specifically binds the catalase or a fragment thereof, preferably from *H. pylori*. However, the invention also comprises embodiments in which those heavy or light chains are combined with other light or heavy chains, wherein the binding properties may essentially be maintained or improved. Corresponding methods are known in the prior art. Particularly preferred antibodies have in the variable regions of the light and heavy chains the amino acid sequences shown in Figures 1 and 2 or the regions are coded by the DNA sequences shown therein.

In a preferred embodiment, the following steps are carried out with the stool sample before incubation with the antibodies: the stool sample is suspended in a sample buffer at a ratio of 1:3 to 1:25, preferably about 1:15. An example of a sample buffer is 150 mM PBS, 0.1% SDS.

In another preferred embodiment, the formation of the at least one antigen-receptor complex/antigen-receptor/receptor-mixture complex in step (b) is detected by means of an immunochromatographic method.

In a particularly preferred embodiment of the method of the invention, in the immunological method, the same receptor is used for both binding to the solid phase and for detecting the epitope. While the catching receptor can be bound to a solid phase, e.g. nitrocellulose, in unmodified form, the receptor used for detection may optionally be labelled.

In a further embodiment the catching receptor can be in biotinylated form and can be bound by way of streptavidin immobilised on the solid phase thereto.

On the other hand the catching receptor may not be biotin-labelled and the epitope of the microorganism, preferably the bacterial epitope, may be detected via a third biotin-labelled receptor, that receptor preferably being an antibody, fragment or derivative thereof or an aptamer, or a species-specific or Ig class-specific antibody or a corresponding aptamer. That third biotin-labelled receptor specifically binds the catching receptor and the analyte-receptor complex is bound by way of the third biotin-labelled receptor to the test line which in this embodiment comprises immobilised streptavidin.

Colloidal gold, selenium, coloured polystyrene or latex particles, carbon particles and disperse dyes (known to the man skilled in the art from the state of the art) can be used as labelling for the receptor used for detection.

On the other hand, as already mentioned, the receptor used for detection may also not be labelled and thus the epitope of the microorganism may also be detected by way of a third labelled receptor which is directed against that non-labelled receptor, that receptor preferably being an antibody, fragment or derivative thereof or an aptamer, which can be a species-specific or Ig class-specific antibody or a corresponding aptamer.

In a particularly preferred embodiment the labelling is colloidal gold.

Labellings of that nature are known from the state of the art, see for example Harlow and Lane, ibid. The same applies to aptamers. The above-described embodiment is particularly suitable for detecting the catalase which may optionally also be present as a tetramer after passage through the intestine. It will be appreciated that in this embodiment combinations of antibodies, fragments, derivatives and aptamers can also be used, e.g. combinations of antibodies etc. which bind to different epitopes of the same antigen.

In another preferred embodiment of the method of the invention the monoclonal antibody is a murine antibody.

In addition, in another preferred embodiment the receptors are fixed to a support.

When carrying out routine checks, it is of particular advantage to fix the receptors, preferably the antibodies, fragments or derivatives thereof or the aptamers to a support. Moreover the combination antibody-support/aptamer-support may be packaged as a tool set or in the form of a kit.

In a particularly preferred embodiment the material of the support is a porous support material.

In another particularly preferred embodiment the support material is a test strip.

In addition in a preferred embodiment the support material consists of cellulose or a cellulose derivative.

The mammal whose stool can be analysed by means of the method of the invention may be an animal, e.g. a domestic animal such as a cat or a dog, a useful animal such as a pig or another kind of animal such as a mouse, a tiger or a ferret.

In a preferred embodiment, the mammal is a human.

Furthermore, the invention relates to a monoclonal antibody, a fragment or derivative thereof having a V-region which has a combination of the aforementioned CDRs or which is produced by one of the aforementioned hybridomas.

In that respect preferred monoclonal antibodies, fragments or derivatives are those which have at least one of the V-regions shown in Figures 1 and 2 or Figures 3 and 4. Preferably, those antibodies have two of the V-regions shown in Figures 1 and 2 or Figures 3 and 4. Moreover those V-regions are preferred to be coded by the DNA sequences shown in Figures 1 and 2 or Figures 3 and 4.

In a particularly preferred embodiment of the invention the monoclonal antibody, the fragment or derivative thereof is a murine antibody or a fragment or derivative thereof or a chimeric, preferably a humanized antibody or a fragment or derivative thereof. The derivative may also be a fusion protein. Furthermore the antibody is preferably labelled, for instance with a colloid, with a labelling comprising gold, selenium, latex, coloured polystyrene, carbon particles or disperse dyes known to the man skilled in the art.

The production of chimeric humanized and human antibodies and of the other derivatives is well known in the state of the art (e.g. Vaughan et al., 1998; Orlandi et al., 1989, Harlow and Lane, ibid.).

The invention also relates to an aptamer which specifically binds the same epitope as the monoclonal antibody, the fragment or derivative thereof. Such aptamers can be produced with methods known in the state of the art.

In addition, the invention relates to an epitope which is specifically bound by one of the above-described monoclonal antibodies, fragments or derivatives thereof or aptamers.

Furthermore, the invention relates to further antibodies, derivatives or fragments thereof, which specifically bind the epitope of the invention. Those antibodies may be for instance monoclonal antibodies which can be produced according to usual methods using the epitope as a hapten/component of an antigen.

Moreover the present invention relates to a diagnostic composition containing at least one receptor, preferably at least one monoclonal antibody, fragments or derivatives thereof or aptamers as defined above, fixed to a support material.

Furthermore, the present invention relates to a test device for detecting at least one epitope as defined above, comprising (a) at least one receptor which is preferably a monoclonal antibody, fragments or

derivatives thereof or an aptamer as defined above, fixed to a support material; (b) a device for preparing and analysing stool samples; and optionally (c) a mixture of receptors as defined above.

As mentioned above the invention further concerns an apparatus for preparing and analysing stool samples as described in WO 98/58587. That apparatus includes sample taking, preparation and test unit (test strip) in one device.

A further subject-matter of the invention is a test device comprising (a) at least one receptor, preferably a monoclonal antibody, fragments or derivatives thereof or an aptamer as defined above, with the receptor being conjugated with colloidal gold, latex particles or other colouring particles, the size of which typically ranges from 5 nm to 100 nm, preferably from 40 nm to 60 nm; (b) a device for preparing and analysing stool samples as described for example in WO 98/58587; and optionally (c) a mixture of receptors as defined above.

Furthermore, the present invention relates to a kit containing (a) at least one receptor which preferably is a monoclonal antibody, fragments or derivatives thereof or an aptamer as defined above, fixed to a support material; optionally also (b) a device for preparing and analysing stool samples as described for example in WO 98/58587; and optionally (c) a mixture of receptors as defined above.

In the Figures:

Figure 1 shows a cloned DNA sequence coding for the V-region of the heavy chain of a monoclonal antibody [HP25.2m/2H10] specific to catalase. The coded amino acid sequence is shown in a single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Figure 2 shows a cloned DNA sequence coding for the V-region of the light chain of a monoclonal antibody [HP25.2m/2H10] specific to catalase. The coded amino acid sequence is shown in a single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Figure 3 shows a cloned DNA sequence coding for the V-region of the heavy chain of a monoclonal antibody [HP25.6m/1B5] specific to catalase. The coded amino acid sequence is shown in a single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Figure 4 shows a cloned DNA sequence coding for the V-region of the light chain of a monoclonal antibody [HP25.6m/1B5] specific to catalase. The coded amino acid sequence is shown in a single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Figure 5 shows the general structure of the rapid test strip; sample application region 1; test or analysis region 3 and absorption region 4. Disposed in the sample application region in the dried condition are the receptors required for the detection operation such as for example specific antibodies for the analyte or the antigen which are labelled with visibly coloured particles, for example colloidal gold or polystyrene, or other binding partners. The test support generally comprises a special test membrane such as for example nitrocellulose. Immobilised on that test membrane are further specific receptors which are directed against the analyte or the antigen, as the test line 6. A filter 2 is disposed between the sample application region and the test region.

Figure 6 shows the structure of the rapid test strip with control line: sample application region 1; test or analysis region 3 and absorption region 4. Disposed in the sample application region in the dried condition are the receptors required for the detection operation for the antigen which are labelled with visibly coloured particles, for example colloidal gold or polystyrene. In a preferred embodiment the sample application region can comprise two mutually superposed conjugate regions which in one region contain the for example gold-labelled receptor and in the other region a biotin-labelled receptor. The test support generally comprises a special test membrane such as for example nitrocellulose. Further specific receptors which are directed against the analyte (antigen) are immobilised on that

test membrane as a test line 6. Streptavidin can be immobilised as the test line in a preferred embodiment. As a function control, a further control or catching line 7, for example a receptor directed against the labelled receptor, can be immobilised on the test membrane. A filter 2 is disposed between the sample application region and the test support.

The examples illustrate the invention.

Example 1: Isolation of *H. pylori* antigens

1.1 Cultivation of *H. pylori*

H. pylori (strain NCTC 11637) was plated in petri dishes on Wilkins Chalgren agar adding 10% horse blood and Amphotericin B, Vancomycin and Cefsoludin (Sigma Chemicals) and incubated in a microaerophile atmosphere (Anaerocult GasPAK, Merck) at 37°C for 1-2 days. The content of 2 dishes was suspended in a 1l-bottle (Schott) in 350 ml of BHIB-medium adding the antibiotics as above, the medium was fumigated for 4-8 min with a gas mixture of 10% CO_2, 5% O_2, 85% N_2 and the bottle was sealed. The culture was shaken on a rotary shaker for 2 days at 37°C. Then the content of the bottle was put in a sterile condition in a 10 l-bottle and filled up with 4.7 l of BHIB-medium. It was then incubated on a rotary shaker for another 2 days at 37°C. Subsequently the whole volume was centrifuged at 5,000 g for 15 min, the supernatant was decanted and the bacteria pellet was weighed. In order to store the pellet, it was resuspended in a physiological saline solution with the addition of 15% glycerine at a ratio of 2:1 (w/v) and frozen at −80°C. In order to check the identity of the cultivated bacteria, a microscopic inspection of the bacteria as well as tests for urease, oxidase and catalase activity were carried out.

Example 2: Preparation of *H. pylori* antigens

Preparation of *H. pylori* lysate

H. pylori bacteria pellet (Example 1) was mixed with PBS, pH 7.5 in a ratio of 1:10 and resuspended on ice. The bacteria cells were subjected to ultrasound on ice with a small probe of an ultrasonic device (Sonifer,

Branson) with an intensity of 25-30% for 10 x 60 s with a break of 60 s each time. The disrupted bacteria cells were centrifuged 2 x 20 min at 4°C and 10,000 rpm (Sorvall, SS34). The supernatant was used as an antigen preparation for the production of polyclonal antisera.

Preparation of *H. pylori* catalase

Frozen bacteria pellet was mixed with disruption buffer (20 mM Tris HC1, pH 7.0, 1 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 0.05% sodium azide and 10% (v/v) isobutanol) in a ratio of 1:2 (w/v) and shaken at room temperature (RT) in an overhead shaker until complete thawing and subsequently shaken for approximately a further 15 min. After centrifuging at 20,000 rpm, 4°C for 20 min, the supernatant was decanted and filtered through a 0.45 µm-filter.

The clear supernatant was diluted with buffer A (20 mM Tris HC1, pH 7.0, 1 mM EDTA, 1 mM PMSF, 0.05% sodium azide) in a ratio of 1:3 and transferred onto a SourceQ column (16/10) (Pharmacia) equilibrated with buffer A. The through-flow of the SourceQ column contained the enzyme catalase and was free of *H. pylori* main antigens such as urease, HSP60 and alkylhydroperoxide reductase.

In order to isolate the catalase, the through-flow of the SourceQ column was subjected to molecular sieve chromatography (Superdex 200) (16/60). The catalase was isolated together with another protein of a size of approx. 150 kDa (neutrophil activating protein, NAP) in about equal shares.

Catalase with a higher purity was obtained when the through-flow of the SourceQ column was brought with a 2 M sodium acetate solution, pH 4.9, to 40 mM sodium acetate and was transferred on a SourceS column (8/28). After washing with a buffer A to remove the proteins that are not bound, the catalase was eluted with buffer B (40 mM sodium acetate, 1 M NaCl, pH 4.9) using a linear NaCl gradient (buffer A plus 0% to 100% of buffer B). Catalase elutes at approx. 370 mM NaCl.

Example 3: Characterisation of the catalase:

Under reducing conditions in SDA PAGE the purified protein had a molecular weight of approx. 58 kDa and a purity of ≥ 90%.

In order to identify the isolated protein a microsequencing procedure was carried out. The protein was cleaved in SDS PAGE gel with LysC protease. The extracted protein mixture was separated via RP-HPLC. The sequence analysis of the LysC peptide resulted in the following amino acid sequence.

ERLHDTIGESLAHVTHK

This sequence is identical to the corresponding LysC peptide from *H. pylori* catalase (Manos J. et al. (1998) Helicobacter 3 (1), 28-38; Genbank accession No AAC16068.1).

Example 4: Production of polyclonal and monoclonal antibodies (pab; mab)

Production of polyclonal antisera:

Polyclonal antisera against *H. pylori* lysate, *H. pylori* lysate with depleted main antigens such as urease, HSP60 and alkylhydroperoxide reductase (cf. Example 2: isolation and purification), *H. pylori* lysate with enriched catalase (for example by adding catalase to the lysate), as well as polyclonal antisera against purified catalase can be obtained by immunising a selected mammal (e.g. mouse, rabbit, goat, etc.) with the corresponding immunogenic preparations containing catalase epitope.

The antibodies can be purified by means of protein A affinity chromatography from sera and can be used as catching antibodies in sandwich ELISA (cf. Example 8) for assessing the suitability of monoclonal antibodies for antigen detection in the stool of patients.

Polyclonal rabbit antisera were generated by pab Productions (Herbertshausen) from *H. pylori* lysate. By means of protein A affinity chromatography polyclonal antibodies were purified from these antisera and used as catching antibodies in sandwich ELISA (cf. Example 8) for

assessing the suitability of monoclonal antibodies for antigen detection in the stool of patients.

Production of monoclonal antibodies:

The monoclonal antibodies were produced according to methods known to the man skilled in the art (Harlow & Lane, 1988; Peters & Baumgarten, 1990).

Immunisation

Antigen preparations produced from *H. pylori* lysate (cf. Example 2) were used for immunising mice (BALB/c x C57 Black, F1 generation, 8-12 weeks old). For basic immunisation 50 µg antigen were emulsified with Freund complete adjuvant (Difco) in a ratio of 1:1 and injected intraperitoneally (200 µl/mouse). In 4-monthly booster shots the mice were given 25 µg antigen each time with Freund incomplete adjuvant. An antiserum as positive control in ELISA (cf. fusion screening) was obtained from blood taken retro-orbitally from the mice.

Fusion

Two days after the last immunisation the spleens of the mice were removed and the spleen cells were fused with the myeloma cells P3x63Ag8.653 (ATCC CRL-1580; Kearney et al., 1979) with polyethylene glycol 4000 in a ratio of 5:1. The fused cells were suspended in HAT medium (cloning medium (=RPMI 1640 medium, 20% FCS, 200 U/ml rhIL-6) with hypoxanthine aminopterin thymidine supplement (Sigma) and plated in 96-well microtitre plates with a cell density of 2-6x10^4 cells/well. The hybridomas were cultivated at 37°C, 5% CO_2, and 95% relative humidity.

Fusion screening by means of direct ELISA

Screening of the antibody-containing culture supernatants from colonised dishes (approx. 10 days after fusion) was carried out in direct ELISA on 96-well microtitre plates (MaxiSorb, Nunc):

The ELISA plates were coated with 2 µg/ml immunisation antigen in carbonate buffer, pH 9.6 (100 µl/well, overnight, 5°C). The coating solution was sucked off and binding sites that were still free were blocked with 2% skimmed-milk powder in PBS (w/v) (200 µl/well, 1 hour, room temperature). After washing the plate twice with PBS, pH 7.3 with 0.025% Tween 20 (v/v), the culture supernatants of the primary clones were pipetted undiluted in the wells (100 µl/well) and the plates were incubated for 1-2 hours at room temperature. Antiserum was used as a positive control, medium as a negative control. After washing again, detection of the bound antibodies was carried out with a peroxidase-labelled secondary antibody (rabbit-anti-mouse Ig-POD (DAKO) in PBS with 0.1% bovine serum albumin, 20 min, room temperature). After washing the plate four times, substrate solution (K-Blue, Neogen or citric acid buffer, pH 4.5, with TMB + H_2O_2) was added. The peroxidase turns the colourless substrate tetramethyl benzidine (TMB, Sigma) into a coloured complex. After 10 min the reaction was stopped by adding 1 N sulphuric acid. Culture supernatants of clones producing antigen-specific antibodies were significantly coloured compared to the colourless negative culture supernatants.

Establishing and cultivating the hybridomas

Positive clones were recloned twice using the principle of limit dilution analysis in order to obtain monoclones (Coller & Coller, 1983). The first recloning operation was carried out in cloning medium with hypoxanthine thymidine supplement (Sigma), the second one in cloning medium. The reclones were in turn examined for antigen specificity by means of direct ELISA. Finally, the recloned clone was adapted to production medium (RPMI 1640 Medium with 5% IgG-reduced FCS) in flat bottles. The cells were cryo-preserved and the culture supernatant was produced for antibody purification.

Example 5: Characterisation of the antibodies from culture supernatant

10 clones were selected from a repertory of 30 specific (producing antibodies against the immunising antigen) clones on the basis of good reactivity to stool samples of patients infected with *H. pylori* in sandwich ELISA (cf. Example 6).

Isotyping

In the culture supernatant isotyping of the monoclonal antibody was carried out with the established clones using the isotyping kit IsoStrip (Roche Diagnostics). That gave 8 type IgG1-clones and one type IgG2a-clone (cf. Table 3).

Western blot

In Western blot the culture supernatants were examined for their ability to specifically recognise the immunising antigen. 15 µg of purified antigen per gel was boiled in reducing sample buffer (Laemmli, 1970) and applied to a 12%-SDS polyacrylamide minigel (8.6 cm x 7.7 cm x 0.1 cm, Biometra). After electrophoretic separation at 25-30 mA the proteins (antigen) were immobilised on a nitrocellulose membrane by means of a semi-dry blot method.

The membrane was blocked with 2% skimmed-milk powder in PBS (30 min, room temperature) and washed three times for 5 mins with TBS/Tween 20 (0.2%). For the following incubation step the membrane was clamped in an Accutran cross blot screening unit (Schleicher and Schuell) using a grid plate with 34 cross channels. In each of the traces that were formed 250 µl of TBS/Tween 20 was provided and in each case 250 µl of the hybridoma culture supernatants to be tested added. Incubation was carried out with shaking for 2 h at room temperature.

After washing three times with TBS/Tween 20, the membrane was incubated for 1 h with the POD-conjugated secondary antibody (rabbit-anti-mouse Ig POD, DAKO). The membrane was washed three times and the

immune complex was visualised by adding the 3,3-diaminobenzidine substrate solution (DAB, Sigma). The antibody-binding protein bands were subsequently visualised by an insoluble peroxidase substrate.

6 hybridoma culture supernatants exhibited a band that corresponds to the catalase (58 kDa), 3 supernatants were negative in Western blot but exhibited a positive reaction with native antigen in ELISA. They are likely to recognise a conformation epitope. Table 2 shows a summary of results.

Example 6: Screening of mab culture supernatants on patient samples (mixed polyclonal/monoclonal system)

Those monoclonal antibodies which exhibited positive antigen recognition in fusion screening by means of direct ELISA were analysed as culture supernatants in sandwich ELISA as to their patient recognition and antigen detection limit.

As internal development samples, stool samples were available, the infection status of which (groups 0 and 4) was determined by means of histological investigation and/or ^{13}C urea breath test. In the case of patients of group 0 an infection with *H. pylori* could be reliably excluded, in the case of patients of group 4 an infection could be certainly detected.

The ELISA plates (MaxiSorb; Nunc) were coated overnight at 5°C with 100 µl of a solution of a polyclonal rabbit-anti-catalase antibody or polyclonal rabbit-anti-*H. pylori* antibody (pab; approx. 20 µg IgG/ml 0.1 M carbonate buffer, pH 9.5). In order to saturate the binding sites that were still free, 200 µl of 150 mM PBS pH 7.2 with 0.2% fish gelatine (w/v) was pipetted per well and incubated at room temperature for 30 min. Then the ELISA plate was washed twice with 250 µl PBS adding 0.025% Tween 20 (washing buffer 1). Human stool was suspended with 150 mM in a ratio of 1:10 (w/v) with the addition of 2% skimmed-milk powder and 1 mM EDTA.

For determining the antigen detection limit an *H. pylori*-negative stool suspension was mixed with 50 ng/ml catalase (see Example 2) and diluted with an *H. pylori*-negative stool suspension in 1:2 steps. 100 µl of

the stool suspension per well was incubated for one hour (double determination in the case of patient samples). The plate was washed 4 times with washing buffer 2 (PBS with 0.2% Tween 20). Then, 100 µl culture supernatant of hybridomas (1:5 diluted in PBS) was added and incubated at room temperature for 60 min. The bound antibodies are detected by adding a conjugated secondary antibody (rabbit-anti-mouse IgG-POD, DAKO). The peroxidase POD turns the colourless substrate tetramethylbenzidine (TMB, Sigma) into a blue product. After 5 to 10 minutes, or as soon as the negative control exhibited a light blue coloration, the reaction was stopped by adding 1 N sulphuric acid (100 µl/well). The intensity of the colour reaction was measured in the ELISA reader (MWG Spektral). Measurement was carried out at 450 nm against the reference wavelength of 620 nm. Before the detection antibody or the substrate solution was added, the ELISA plate was washed three to four times in each case with washing buffer 1.

The lowest concentration at which extinction greater than or equal to double the control (*H. pylori*-negative stool sample without antigen addition) was still detected was determined to serve as the detection limit.

Table 1: HP25.2m/2H10: sensitivity and specificity in sandwich ELISA with patient samples

stool sample	Patient infection status	catching ab: pab against HP detection ab: HP25.2m/2H10 (culture supernatant) $OD_{450-620}$	evaluation cut off: 0.1: $OD_{450-620}$=0.1
CX0010	POSITIVE	0.25	positive
CX1014	POSITIVE	0.75	positive
CX1029	POSITIVE	0.18	positive
CX1038	POSITIVE	0.09	negative
CX1052	POSITIVE	0.11	positive
CX2008	POSITIVE	0.63	positive
CX2009	POSITIVE	0.32	positive

CX2016	POSITIVE	0.07	negative
CX2019	POSITIVE	0.59	positive
CX2029	POSITIVE	0.52	positive
CX2013	POSITIVE	0.04	negative
CX294-1	POSITIVE	0.14	positive
CX3098	POSITIVE	0.13	positive
CX3146	POSITIVE	0.05	negative
CX3148	POSITIVE	0.08	negative
CX3234	POSITIVE	0.18	positive
CX4003	POSITIVE	0.17	positive
CX4006	POSITIVE	0.25	positive
CXT001	POSITIVE	0.23	positive
CXT002	POSITIVE	0.53	positive
CXT003	POSITIVE	0.12	positive
CXT004	POSITIVE	0.03	negative
CXT005	POSITIVE	0.03	negative
CXT006	POSITIVE	0.31	positive
CXT007	POSITIVE	0.08	negative
CX1008	NEGATIVE	0.29	positive
CX1031	NEGATIVE	0.08	negative
CX1049	NEGATIVE	0.7	positive
CX1051	NEGATIVE	0.09	negative
CX0142	NEGATIVE	0.03	negative
CX0185	NEGATIVE	0.03	negative
CX0189	NEGATIVE	0.08	negative
CX0193	NEGATIVE	0.03	negative
CX2010	NEGATIVE	0.08	negative
CX2018	NEGATIVE	0.09	negative
CX0220	NEGATIVE	0.03	negative

CX0231	NEGATIVE	0.03	negative
CX0258	NEGATIVE	0.02	negative
CX3008	NEGATIVE	0.09	positive
CX3011	NEGATIVE	0.08	negative
CX3033	NEGATIVE	0.07	negative
CX3035	NEGATIVE	0.09	negative

ab: antibody; HP: *H. pylori*

The monoclonal antibody HP25.2m/2H10 exhibited in the sandwich ELISA with patient samples a sensitivity of 68% (out of 25 positive samples 17 were detected) and a specificity of 82% (out of 17 samples 14 were correctly detected).

Table 2: Characterisation of the monoclonal antibodies against catalase

fusion/clone	isotype	WB (ag)	NWG (ng/ml)	stool samples that were correctly detected	
				pos.samples	neg.samples
HP25.2m/2H10	IgG2a, K	+	1.5	17 out of 25	14 out of 17
HP25.6m/IG4	IgG1, K	+	1.5	4 out of 5	2 out of 2
HP25.6m/IB5	IgG1, K	+	3-6	3 out of 5	2 out of 2
HP25.6m/IH4	IgG1, K	+	3-6	2 out of 5	2 out of 2
HP25.6m/4E3	IgG1, K	+	6	2 out of 5	2 out of 2
HP25.6m/1A5	IgG1, K	+	6	2 out of 5	2 out of 2
HP25.6m/5E4	IgG1, K	-	1.5	1 out of 5	2 out of 2
HP25.6m/4A12	IgG1, K	-	1.5	1 out of 5	2 out of 2
HP25.6m/5F4	IgG1, K	-	1.5	1 out of 5	2 out of 2

ag: antigen; WB: Western blot; NWG: detection limit

Results:

Table 2 summarises the results of isotype determination, Western blot analyses, determination of detection limits and patient recognition for the monoclonal antibodies against catalase. In the mixed

polyclonal/monoclonal sandwich ELISA system the mab HP25.2m/2H10 exhibited a sensitivity of 68% and a specificity of 82%. An improvement in sensitivity and specificity was exhibited by using purified mab (instead of culture supernatant) in a purely monoclonal ELISA system.

In this case, either a monoclonal antibody directed against the same epitope of the antigen or two different monoclonal antibodies directed against different epitopes of the same antigen (see Example 8) can be used as catching and detection antibodies.

Example 7: Purification of monoclonal antibodies from hybridoma culture supernatants

The purification of mab from serum-free hybridoma culture supernatants is carried out by means of modified protein-G affinity chromatography (Pharmacia Biotech, 1994). The filtered (0.45 µm) culture supernatants were conducted directly over a protein G matrix. Protein detection in the through-flow or in the eluate was carried out by way of measuring the optical density at 280 nm. After washing with 150 mM PBS, pH 7.2, until the detector background value was attained, elution was conducted with 0.1 M glycine/HCl, pH 3.3. The protein G matrix was regenerated with 0.1 M glycine/HCl, pH 2.7.

Example 8: Characterisation of the purified monoclonal antibodies and antibody selection for the test

Those antibodies which exhibited best stool sample detection in measurement from the culture supernatant were further characterised in the purified condition. On the one hand the affinity constants were determined by means of surface plasmon resonance. In addition the binding regions of the antibodies were mapped (epitope mapping). Finally a selection of suitable antibody pairs was effected on the basis of stool samples in sandwich stool ELISA and in the rapid test.

Characterisation of antibody-antigen interactions by means of surface plasmon resonance spectroscopy (SPR spectroscopy)

By means of SPR spectroscopy it is possible to determine the affinity constants of the monoclonal antibodies. Suitable antibodies for the development of ELISA and rapid test can be found in that way.

Conducting surface plasmon resonance spectroscopy on the Pharmacia BIAcore

All steps were carried out on a Pharmacia Biacore Processing Unit CA 186 according to the manufacturer's instructions (BIAcore Methods Manual).

Catalase was immobilized by way of amine coupling on the dextrane matrix of the BIAcore CM5 sensor chip. For activation of the dextrane matrix 45 µl of a 1:1 mixture of 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) solution was passed over the sensor chip at a flow rate of 5 µl/min. Then catalase (35 µl; 50 µg/ml in a 10 mM sodium acetate pH 5.0) was bound to the dextrane matrix. Remaining NHS esters were deactivated with 1 M ethanolamine (35 µl). Catalase that was not covalently bound to the dextrane matrix was removed by regenerating the sensor chip with HCl (10 mM; 15 µl).

By adding the catalase-specific monoclonal antibodies, they were made to react with immobilised catalase and the mass attachment to the detector was measured. Antibody solutions in different concentrations ranging from 20 to 670 nM were used. They were injected over the catalase immobilised on the sensor chip CM5 at a flow rate of 25 µl/min in each case.

The values for the rate constants of adsorption (k_{on}) and desorption (k_{off}) of the antibody can be calculated from the time course of the resonance signal (BIAevaluation software 3.0). Of the 6 monoclonal antibodies which were tested 4 exhibited very good affinities K_D>5E-10 (Table 3).

Table 3: Results of affinity determination of the monoclonal antibodies against catalase

Mab	$k_{on}[M^{-1}\ s^{-1}]$	$k_{off}[s^{-1}]$	$K_D[M]$
HP25.2m/2h10	1.44E+05	3.90E-05	2.71E-10
HP25.6m/1G4	1.41E+05	2.52E-05	1.79E-10
HP25.6m/1B5	5.67E+04	3.86E-05	6.81E-10
HP25.6m/1H4	7.12E+04	4.12E-05	5.79E-10
HP25.6m/4E3	4.92E+04	5.96E-05	1.21E-09
HP25.6m/1A5	3.91E+04	4.77E-05	1.22E-09

$K_D = k_{off} : k_{on}$

Epitope mapping of the monoclonal antibodies against catalase

Epitope mapping was carried out by Pepscan Systems (Netherlands). A peptide bank (30-mers with an overlap of 27 amino acids) of the catalase was produced on plastic cards and incubated with the antibodies. The ascertained epitopes (peptides to which antibodies have bound) are set out in Table 4. HP25.2m/2H10 exhibited non-specific peptide recognition, that is to say that antibody very probably binds to a discontinuous structure epitope. Besides the main recognition region (see Table 4) HP25.6m/1B5 exhibited further non-specific peptide bindings which exhibited the co-operation of a structure component. If the ascertained epitopes are transferred to the structure of E.coli catalase (Bravo et al., 1999), it is found that the antibodies HP25.6m/1B5, 1A5 4E3, 1G4 and 1H4 bind in the enzyme centre (amino acid 190 – 360), a region which is in the proximity of the catalytic domain.

Table 4: Results of epitope mapping of the catalase mab

map	**recognised epitope**
HP25.2m/2H10	discontinuous
HP25.6m/1B5	EGNWDLVGNNTPVFFIRDAIKFPDFIHTQKRDPQTN
HP25.6m/4E3	IARGDYPKWLSTQVMPEEDAKKYRFHPFDVTK
HP25.6m/1A5	IARGDYPKWLSTQVMPEEDAKKYRFHPFDVTK
HP25.6m/1H4	SRGDYMQNGYYGSLQNYTPSSLPGYKEDKS
HP25.6m/1G4	1. EEAAEIRKHDPDSNQRDLFDAIARGDYPKW 2. DDSDYYTQPGDYYRSLPADEKERLHDTERLH DTIGESLAHYTHKAEIVDKQLEHFKKA

Overlapping recognition regions of the antibodies are underlined.

Ascertaining suitable antibody pairs by means of patient stool

The antibodies against catalase were firstly titrated out against each other. Then patient stool samples were tested with the ELISA systems optimised in that way, and the detection limits of catalase in human zero stool determined (Table 5).

Structure of the sandwich ELISA

The ELISA plates (MaxiSorb; Nunc) were coated with 100 µl of a mab solution in 0.1 M carbonate buffer, pH 9.5 for 1 hour at 37°C. In order to block the binding sites that were still free, 200 µl 150 mM PBS with 0.2% fish gelatine (w/v) was pipetted per dish and incubated at room temperature for 30 min. Subsequently they were washed twice with 250 µl washing buffer 1 (PBS with 0.025% Tween). Human stool was suspended with 150 mM PBS in a ratio of 1:10 (w/v) with the addition of 2% of skimmed-milk powder and 1 mM EDTA. In order to determine the antigen detection limit purified *H. pylori* catalase was added in known concentrations to the stool suspension of an *H. pylori*-negative patient (zero stool). The stool sample suspensions were centrifuged off at 7,000 g for 5 min. 100 µl of the supernatant per well was incubated for one hour.

The samples were applied as double values. The plate was then washed four times with washing buffer 2 (250 µl PBS with the addition of 0.2% Tween). Then 100 µl of a solution of biotin-coupled mab in PBS; 0.1% BSA was added and incubated at room temperature for 60 min. The bound antigen/antibody complexes are detected by adding a conjugate of streptavidin with POD (Dianova). In the next step the POD then turns the colourless substrate TMB (Sigma) into a blue product. After five to ten minutes or as soon as the negative control exhibited a light blue coloration the reaction was stopped by adding 1N sulphuric acid (100 µl/well). The intensity of the colour reaction was measured in the ELISA reader (MWG Spektral). Measurement is carried out at 455 nm against the reference wavelength of 620 nm.

HP25.2m/2H10 was found to be a suitable detection antibody for the combination with all other tested antibodies. On the basis of the affinity data HP25.6m/1B5, 1G4 and 1H4 were tested as catching antibodies in the rapid test. In that respect HP25.6m/1B5 was found to be the best catching antibody.

Table 5: Results of pair finding of the monoclonal antibodies against catalase

	Catching antibodies				
Biotinylated detector antibody	25.2m/2H10	25.6m/1B5	25.6m/1G4	25.6m/1A5	25.6m/1H4
25.2m/2H10	N: 0.03 G4: 7-8 G0: 2	0.1 7 2	0.03 8 2	0.1 7 2	0.03 8 2
25.6m/1B5	N: 0.01 G4: 8 G0: 2	0.1 7 1	0.1 5 2	0.03 7 2	0.3 8 2
25.6m/1G4	N: 0.03 G4: 6-8 G0: 1-2	0.1 7 2	0.1 8 4	0.1 8 2	0.1 8 2
25.6m/1A5	N: 0.03 G4: 6-7 G0: 2	0.1 7 2	0.3 5 2	0.1 7 2	0.3 8 2
25.6m/1H4	N: 0.1 G4: 8 G0: 3	0.3	0.1 4-7 2	0.3 7 2	0.1 8 2

Patient recognition (detection of 8 critical G4- and 4 G0-patient samples)
N = detection limits [ng/ml] of the catalase in zero stool

Example 9: Production of conjugates for use in immunochromatographic rapid tests

Coupling of monoclonal antibodies to biotin

The monoclonal antibodies were coupled to biotin after the cleaning operation. Coupling was effected using known methods (Harlow & Lane, 1988). The monoclonal antibodies were conjugated at a concentration of about 1 – 2 mg/ml. Before coupling the antibodies were rebuffered by

dialysis in 0.1 M sodium acetate buffer, pH 8.3 or 0.1 M sodium hydrogen carbonate buffer; pH 8.3. For each 1 mg of antibodies 50 µg of N-hydroxysuccinimidobiotin (NHS-d-biotin; Sigma) was added in DMSO and mixed. The mixture was incubated for 1 h at room temperature. After that the biotinylated antibodies were freed of uncoupled NHS-d-biotin by extensive analysis against 0.15 M PBS, 0.05% NaN_3, pH 7.5.

Coupling of monoclonal antibodies to colloidal gold

Coupling of monoclonal antibodies to colloidal gold was effected in accordance with known standard methods (Frens. 1973; Geoghegan und Ackerman, 1977; Slot et al., 1985). Gold with a particle size of 40 nm, OD (520 nm) = 1 (British BioCell, Cardiff, England) was adjusted to pH 9 with 0.1 M K_2CO_3. Purified mab was dialysed against 2 mM borate buffer, pH 9.2, and diluted to a concentration of 0.1 mg/ml. For coupling purposes 2 ml of the mab solution was added dropwise to 20 ml of the colloidal gold solution with rapid agitation and incubated for 30 min at room temperature. The optimum IgG concentration and the suitable pH-value for coupling were individually determined for each mab. 10 µg of IgG/ml gold colloid can be used as a guideline. For stabilisation of the gold IgG conjugate 2 ml bovine serum albumin was added in a concentration of 10% and incubated for a further 5 min. Gold colloid that was not coupled to IgG and free IgG were then separated off by centrifuging. For that purpose the coupling composition was centrifuged for 30 min at 15,000 rpm (Sorvall, SS-34) and the clear supernatant was removed by suction removal. The gold IgG conjugate, which settled in the form of a loose sediment coloured dark red at the bottom of the centrifuge tube, was taken up in 2 ml of 20 mM Tris, pH 8.2 with the addition of 1% bovine serum albumin and 0.05% NaN_3.

Example 10: Immunochromatographic rapid test

An immunochromatographic test using the sandwich principle was constructed with the antibody pair HP25.2m/2H10 and HP25.6m/1B5. As diagrammatically shown in Figures 5 and 6 that test comprises a sample

application region 1, a filter 2, a test or analysis region 3 and an absorption region 4.

The purified mab HP25.2m/2H10 was coupled to gold as the signal-forming immunoreagent (British BioCell, Cardiff, England). The mab-gold conjugate was diluted in deionised water with the addition of 5% saccharose (Sigma, Deisenhofen) to an OD (520 nm) of 6 and applied to a conjugate fleece of glass fibre (Pall, Dreiech). The conjugate fleece was then subjected to vacuum drying.

As the test region (Figure 6, 3) nitrocellulose was coated with a flow rate of 95 – 175 sec/4 cm (Millipore, Bedford, MA, USA) with the immunoreagents forming the test and control line. A special applicator device for test strips was used for that purpose (Imagene, Hanover, NH, USA). Purified mab HP25.6m/IB5, 1 – 4 mg/ml, in phosphate buffer pH 7.4 in a concentration of 1 – 2 µg/cm was applied as the test line (Figure 6, 6). As the control line (Figure 6, 7) a polyclonal anti-mouse-antibody (Dianova, Hamburg) was applied in a concentration of 0.1 – 0.3 µg/cm.

The coated nitrocellulose and the coated conjugate fleece were then glued together with the further components of the test strip onto polyester supports (G&L, Santa Clara, CA, USA) and cut into 5 mm wide individual strips. Glass fibre materials (Pall, Dreiech; Whatman, Maidstone, England) in a width of 1 – 2 cm were used as filter (Figure 6, 2). Absorbent cellulose or cellulose-glass fibre materials in a width of 2 – 3 cm were used for the absorption region (Figure 6, 4) (Pall, Dreich; Schleicher & Schuell, Dassel; Whatman, Maidstone, England).

Example 11: Immunochromatographic rapid test using streptavidin as the test line

As a departure from the rapid test described in Example 10 the mab HP25.6m/1B5 used there as the test line was coupled to biotin and dried onto a second conjugate fleece of the sample application region.

Recombinant streptavidin (Roche, Mannheim) in a concentration of 10-20 mg/ml in phosphate buffer, pH 7.4, was applied by coating as the test line.

In that sandwich structure both antibody conjugates are mobile and during implementation of the test move over the test strip. In the presence of antigen the entire sandwich complex is already formed during such movement, and is caught at the test line by way of the binding of biotin to streptavidin.

Example 12: Detection of *H. pylori* in the human stool by means of an immunochromatographic rapid test

Patient samples

200 stool samples from patients from ten different hospitals or gastroenterological surgeries in Germany were available for evaluation of the immunochromatographic rapid test. The samples originated both from patients who had no troubles and diseases of the gastrointestinal tract and also from patients who were subjected to an examination because of troubles or diseases of the gastrointestinal tract. The infection status of the patients was determined by means of ^{13}C urea breath test or by means of histological investigation of a gastric biopsy sample. Patients who showed contradictory results in these two methods accepted as the gold standard were not included in the evaluation. The infection status of the patients tested was not known to the laboratory staff.

Test implementation

To carry out the rapid test the stool samples were dissolved in a ratio of 1:15 in a sample buffer and 500 µl of the sample fluid applied to the application zone (sample application region) of the test strip. The test was visually evaluated after 15-20 minutes. The test signal at the test line was classified as present (test result positive) or absent (test result negative). The operation of reading off the test result was effected in each case independently of each other by three persons who had no qualification as

laboratory staff. The tests were also evaluated by the laboratory staff half quantitatively with 0 (negative), 1 (weakly positive) and 2 (greatly positive).

Table 6 shows the results which were achieved by means of the stool rapid test in comparison with the two reference methods in an evaluation on a total of 200 patient samples. Of the total of 100 *H. pylori*-positive samples 95 were positively tested correctly while in the case of 5 samples a falsely negative result occurred. Of the total of 100 *H. pylori*-negative samples 94 samples were negatively tested correctly while a falsely positive result occurred in 6 samples. Sensitivity and specificity of the rapid test were 95% and 94% in comparison with the reference methods.

Table 6: Comparison of the test results of the rapid test and the gold standard in the investigation of a total of 200 stool samples

number of the sample	result of the ^{13}C breath test	result of the gastric biopsy	result of the (semi-quantitative) *H. pylori*-rapid test
1001	n.d.	negative	0
1002	n.d.	negative	0
1007	n.d.	negative	0
1008	n.d.	negative	0
1010	n.d.	negative	0
1012	n.d.	negative	0
1017	n.d.	negative	0
1021	n.d.	negative	0
1022	n.d.	negative	0
1024	n.d.	negative	0
1025	n.d.	negative	0
1027	n.d.	negative	0
1030	n.d.	negative	0
1031	n.d.	negative	0

1032	n.d.	negative	0
1034	n.d.	negative	0
1035	n.d.	negative	0
1040	n.d.	negative	0
1046	n.d.	negative	0
2002	n.d.	negative	0
2006	n.d.	negative	0
2007	negative	n.d.	0
2010	n.d.	negative	0
2012	negative	n.d.	0
2013	negative	n.d.	0
2014	negative	n.d.	0
2015	n.d.	negative	1
2017	negative	negative	0
2018	negative	negative	0
2023	n.d.	negative	0
2024	negative	n.d.	0
2028	n.d.	negative	0
2033	negative	negative	0
2034	negative	negative	0
2043	n.d.	negative	0
3123	negative	n.d.	0
3213	n.d.	negative	0
3224	negative	n.d.	0
3225	n.d.	negative	0
4004	n.d.	negative	0
5004	n.d.	negative	0
5007	n.d.	negative	0
5008	n.d.	negative	0

5009	n.d.	negative	0
5010	n.d.	negative	0
5012	n.d.	negative	0
5013	n.d.	negative	0
5017	n.d.	negative	0
5018	n.d.	negative	0
5019	n.d.	negative	0
5020	n.d.	negative	0
5021	n.d.	negative	0
5022	n.d.	negative	0
5024	n.d.	negative	0
5025	n.d.	negative	0
5027	n.d.	negative	0
5028	n.d.	negative	0
5030	n.d.	negative	0
5031	n.d.	negative	2
5033	n.d.	negative	0
5035	n.d.	negative	0
5036	n.d.	negative	0
5040	n.d.	negative	0
5042	n.d.	negative	0
5046	n.d.	negative	0
5052	n.d.	negative	0
5056	n.d.	negative	1
5057	n.d.	negative	0
5060	n.d.	negative	1
5063	n.d.	negative	0
5064	n.d.	negative	0
5066	n.d.	negative	0

5067	n.d.	negative	0
5068	n.d.	negative	0
6002	n.d.	negative	0
6005	n.d.	negative	0
6008	n.d.	negative	0
6009	n.d.	negative	0
6017	n.d.	negative	0
6019	n.d.	negative	0
6024	n.d.	negative	0
6026	n.d.	negative	0
6029	n.d.	negative	0
6033	n.d.	negative	0
6038	n.d.	negative	0
6039	n.d.	negative	0
7005	n.d.	negative	0
7006	n.d.	negative	2
7009	n.d.	negative	0
7013	n.d.	negative	0
8004	n.d.	negative	0
8047	n.d.	negative	0
9004	n.d.	negative	0
9005	n.d.	negative	0
9010	n.d.	negative	0
9011	n.d.	negative	0
9012	n.d.	negative	0
9013	n.d.	negative	0
9015	n.d.	negative	1
9019	n.d.	negative	0
213	n.d.	positive	1

444	n.d.	positive	1
1003	n.d.	positive	1
1013	n.d.	positive	2
1014	n.d.	positive	1
1015	n.d.	positive	2
1028	n.d.	positive	1
1029	n.d.	positive	2
1037	n.d.	positive	1
2005	positive	n.d.	2
2008	n.d.	positive	2
2009	positive	n.d.	2
2016	n.d.	positive	2
2029	positive	positive	2
2032	positive	positive	2
2035	n.d.	positive	2
2039	positive	positive	2
2040	n.d.	positive	2
2041	positive	positive	2
2042	positive	positive	2
3146	positive	n.d.	2
3219	positive	positive	2
3220	positive	positive	2
3231	positive	positive	2
3234	positive	positive	2
3241	positive	positive	1
3570	positive	n.d.	2
4003	n.d.	positive	2
4005	positive	positive	1
4006	n.d.	positive	2

4018	n.d.	positive	2
4019	n.d.	positive	2
4020	n.d.	positive	2
5001	n.d.	positive	2
5006	n.d.	positive	2
5029	n.d.	positive	2
5039	n.d.	positive	2
5048	n.d.	positive	2
5050	n.d.	positive	1
5053	n.d.	positive	2
5055	n.d.	positive	2
5058	n.d.	positive	2
5061	n.d.	positive	2
5069	n.d.	positive	1
5072	n.d.	positive	2
5075	n.d.	positive	2
5076	n.d.	positive	2
5078	n.d.	positive	2
5090	n.d.	positive	2
5092	n.d.	positive	2
5100	n.d.	positive	2
5150	n.d.	positive	0
6001	n.d.	positive	2
6004	n.d.	positive	2
6013	n.d.	positive	1
6014	n.d.	positive	2
6015	n.d.	positive	2
6018	n.d.	positive	2
6020	n.d.	positive	1

6022	n.d.	positive	2
6027	n.d.	positive	2
6040	n.d.	positive	2
6050	n.d.	positive	2
6052	n.d.	positive	2
6064	n.d.	positive	2
6065	n.d.	positive	2
7001	n.d.	positive	1
7002	n.d.	positive	2
7003	n.d.	positive	1
7020	n.d.	positive	0
8026	n.d.	positive	0
8033	n.d.	positive	2
9001	n.d.	positive	2
9002	n.d.	positive	2
9003	n.d.	positive	2
9006	n.d.	positive	2
9007	n.d.	positive	1
9008	n.d.	positive	2
9009	n.d.	positive	0
9014	n.d.	positive	2
9017	n.d.	positive	2
9018	n.d.	positive	2
9022	n.d.	positive	2
T01	positive	n.d.	2
T02	positive	n.d.	2
T03	positive	positive	2
T04	positive	positive	1
T05	positive	positive	1

T07	positive	positive	1
T09	positive	n.d.	2
T10	positive	n.d.	1
T13	n.d.	positive	2
T53	positive	n.d.	2
T58	positive	n.d.	1
T64	positive	n.d.	2
T67	positive	n.d.	1
T68	positive	n.d.	2
T70	positive	n.d.	1
T77	positive	n.d.	0
T88	positive	n.d.	2

n.d.: not determined; 0: negative; 1: weakly positive; 2: greatly positive

(n=200)

method		gold standard	
		positive	negative
H. pylori rapid test	positive	95	6
	negative	5	94

Sensitivity: 95%

Specificity: 94%

Example 13: Cloning and sequence determination of the functional variable regions of immunoglobulins from hybridoma cell lines

Total RNA was isolated from antibody-producing hybridoma cell lines according to Chomczynski (Chomczynski. 1987). The corresponding cDNA was synthesised using standard methods (Sambrook et al., 1989).

The DNA regions coding the kappa-light chain as well as the heavy chain Fd segment (VH or CH1) of the respective antibodies were amplified by means of PCR. The oligonucleotide primer set stated in Table 7 was used, the cDNA isolated from the single hybridoma cell lines served as a template.

The primer set used leads to a 5'-*XhoI* and a 3'-*SpeI* cleavage site in the heavy chain Fd fragments as well as to a 5'-*SacI* and a 3'-*XbaI* cleavage site in the kappa-light chains. For PCR amplification of the DNA fragments coding the heavy chain Fd, 11 different 5'-VH primers (MVH 1-8 and MULH 1-3) were each combined with the 3'-VH primer MIgG2a. For the amplification of the DNA fragments which code for the kappa-light chains, 11 different 5'-VK primers (MUVK 1-7 and MULK 1-4) were each combined with the 3'-VK primer 3' MUCK.

The following temperature program was used in all PCR amplification procedures: denaturation at 94°C for 30 s, primer attachment at 52°C for 60 s, polymerisation at 72°C for 90 s. This program was maintained for 40 cycles, followed by a final completion of the fragments at 72°C for 10 min.

The results of the PCR amplification procedures were separated by means of agarose gel electrophoresis and DNA bands of the expected molecular weight were isolated.

For the antibodies 25.2m/2H10 the isolated bands were then subjected to restriction digestion using the enzymes XhoI and SpeI (heavy chains), and SacI and XbaI (light chains) respectively and the fragments obtained were cloned into the plasmid vector Bluescript KS (Strategene) after the vector had first been cleaved with the restriction enzymes XhoI and SpeI, and SacI and XbaI respectively.

Subsequently, plasmid preparations of the cloned heavy and light chain fragments were sequence-analysed. Sequences were chosen which code for functional variable regions of the immunoglobulin the heavy and light chains (VH or VL). In that way it was possible to identify exactly one

functional VH and one functional VL region for each hybridoma cell line. Figure 1 and Figure 2 show the functional VH and VL sequences. The first four amino acids of the VH region were completed by recloning. Cloning and sequencing were carried out using standard methods (Sambrook et al., 1989).

For the antibody 25.6m/1B5 the isolated bands were then directly sequenced and a functional light and a functional heavy chain identified. The heavy chain Fd fragment and the light chain were then subjected to a restriction digestion using the enzymes XhoI and SpeI (heavy chain), and SacI and XbaI (light chain) and the fragments obtained were cloned into the plasmid vector pBSIIIHisEx (Connex) after it had firstly been cleaved with the restriction enzymes *Xho* I and *Spe* I, and *Sac* I and *Xba* I and sequenced again.

In that way it was possible to identify exactly one functional VH and one functional VL region for that hybridoma cell line. The functional VH and VL sequences are shown in Figure 3/Figure 4. In the case of the VH and VL sequences the mature N-termini are illustrated, as were ascertained by the sequencing operation by means of leader primers. Cloning and sequencing were carried out using standard methods (Sambrook et al., 1989).

Table 7: List of the primers used for the PCR amplification of the functional variable regions of heavy and light immunoglobulin-chains (orientation 5'-3')

MVH1	(GC)AG GTG CAG CTC GAG GAG TCA GGA CCT
MVH2	GAG GTC CAG CTC GAG CAG TCT GGA CCT
MVH3	CAG GTC CAA CTC GAG CAG CCT GGG GCT
MVH4	GAG GTT CAG CTC GAG CAG TCT GGG GCA
MVH5	GA(AG) GTG AAG CTC GAG GAG TCT GGA GGA
MVH6	GAG GTG AAG CTT CTC GAG TCT GGA GGT
MVH7	GAA GTG AAG CTC GAG GAG TCT GGG GGA
MVH8	GAG GTT CAG CTC GAG CAG TCT GGA GCT
MULK1	GGG GAG CTC CAC CAT GGA GAC AGA CAC ACT CCT GCT AT
MULK2	GGG GAG CTC CAC CAT GGA TTT TCA AGT GCA GAT TTT CAG
MULK3	GGG GAG CTC CAC CAT GGA GWC ACA KWC TCA GGT CTT TRT A
MULK4	GGG GAG CTC CAC CAT GKC CCC WRC TCA GYT YCT KGT
MIgG2a	GAG AGA GGG GTT CTG ACT AGT GGG CAC TCT GGG CTC
MUVK1	CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT
MUVK2	CCA GTT CCG AGC TCG TGT TGA CGC AGC CGC CC
MUVK3	CCA GTT CCG AGC TCG TGC TCA CCC AGT CTC CA
MUVK4	CCA GTT CCG AGC TCC AGA TGA CCC AGT CTC CA
MUVK5	CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA
MUVK6	CCA GAT GTG AGC TCG TCA TGA CCC AGT CTC CA
MUVK7	CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA
MULH1	GGG CTC GAG CAC CAT GGR ATG SAG CTG KGT MAT SCT CTT
MULH2	GGG CTC GAG CAC CAT GRA CTT CGG GYT GAG CTK GGT TTT
MULH3	GGG CTC GAG CAC CAT GGC TGT CTT GGG GCT GCT CTT CT
3'MUCK	GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A

Literature:

Coller & Coffer, 1983: Coller, H.A., Coller, B.S., Meth. Enzymol. 121:412-417
Harlow & Lane, 1988: Harlow, E., Lane, D., Antibodies: A laboratory manual, Cold Spring Harbour Laboratory, New York
Kearney et al., 1979: Kearney, J. Immunol. 123: 1548-1550
Laemmli, 1970: Laemmli, E.K., Nature 227: 680-685
Peters & Baumgarten, 1990: Peters, J.H., Baumgarten, H. (editor), Monoklonale Antikörper, Springer Verlag, Berlin
Fägerstam et al., 1990: Fägerstam, L.G. et al., J. Mol. Recognit. 3: 208-214
Malmgvist, 1996: Methods 9: 525-532
Eschweiler et al., 1993: Eschweiler, B., et al., Zentralbl. F. Bakt. 280: 73-85 Pharmacia Biotech, 1994: Monoclonal Antibody Purification Handbook
Chomczynski, 1987: Anal. Biochem. 162: 156-159
Sambrook et al., 1989: Molecular cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, second edition
Vaughan et al., 1998: Nature Biotechnology 16: 535-539
Orlandi et al., 1989: Proc. Natl. Acad. Sci USA 86: 3833-3837
Janeway & Travers, 1997: Immunologie, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg
Osborne et al., 1997: Curr. Opin. Chem. Biol. 1: 5-9
Stull and Szoka, 1995: Pharm. Res. 12: 465-483
Frens, 1973: Nat. Phys. Sci. 241, 20-23
Geoghegan and Ackerman, 1977: J. of Histochemistry and Cytochemistry, 25(11), 1187-1200
Slot, 1985: Eur. J. Cell Biol. 38, 87-93
Manos et al.,1998: Helicobacter 3 (1), 28-38
Haque, 1993: J. Infect. Dis. 167: 247-9
Park, 1996: J. Clin. Microbiol. 34: 988-990
Hasan, 1994: FEMS Microbiol. Lett. 120: 143-148
Koopmans,1993: J. Clin. Microbiol. 31: 2738-2744
Machnicka,1996: Appl. Parasitol. 37: 106-110

CLAIMS

1. A method of detecting an infection of a mammal with an acid-resistant microorganism, which includes the following steps:

(a) providing an immunochromatographic test with a sample application region for application of a stool sample of the mammal with an antigen and application of the stool sample,

(b) incubating the stool sample with (i) a first receptor under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the receptor; or (ii) at least two different first receptors under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the two first receptors, and wherein the first receptor according to (i) or the first receptors according to (ii) specifically bind(s) an antigen which at least in some of the mammals has a structure after passage through the intestine that corresponds to the native structure or the structure against which a mammal produces antibodies after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide,

(c) providing a second receptor immobilised on an analysis region, wherein the second receptor binds an antigen-receptor complex according to (b), and

(d) transporting and detecting the formation of at least one antigen-receptor complex according to (b) by accumulation of the antigen-receptor complex at the second receptor in the analysis region.

2. An immunochromatographic test for detecting an infection of a mammal with acid-resistant microorganism, wherein the test is particularly suited or adapted to carrying out a method according to claim 1, comprising:

(a) a sample application region (1, 2) for the application of a stool sample of the mammal with an antigen,

(b) a device for incubating the stool sample with (i) a first receptor (5) under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the receptor; or (ii) two different first receptors under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the two first receptors, and wherein the first receptor according to (i) or the first receptors according to (ii) specifically bind(s) an antigen which at least in some of the mammals has a structure after passage through the intestine that corresponds to the native structure or the structure against which a mammal produces antibodies after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide,

(c) a second receptor (6) immobilised on an analysis region, wherein the second receptor (6) binds an antigen-receptor complex according to (b), and

(d) a transport device (3) which transports the antigen-receptor complex according to (b) to the analysis region with the immobilised second receptor (6) for the accumulation of the analyte-receptor complex.

3. A method or test according to claim 1 or claim 2 wherein the stool sample is suspended prior to the application operation.

4. A method or test according to one of the preceding claims wherein a test strip with an analysis region of cellulose or cellulose derivative is provided and the support material is suitable for transport to occur by way of the capillary forces in the support material.

5. A method or test according to one of the preceding claims wherein the sample application region has a conjugate fleece (1) and a filter (2) which is arranged downstream in the transport direction and which is

suitable for substantially filtering the solid constituents of the stool or the stool suspension.

6. A method or test according to claim 5 wherein the filter (2) has an exclusion size of 1 to 2 µm.

7. A method or test according to claim 5 or claim 6 wherein the filter is made from glass fibre and/or polyester-glass fibre mixes.

8. A method or test according to one of claims 4 to 6 wherein the test strip is arranged fixedly on a polyester support.

9. A method or test according to one of the preceding claims wherein antibodies or antibody conjugates are provided as the first and/or second receptor or receptors.

10. A method or test according to one of claims 4 to 9 wherein the first receptor or receptors (5) is soluble by the suspension and/or the second receptor (6) is or are immobilised or dried onto the test strip non-solubly by the suspension.

11. A method or test according to one of the preceding claims wherein in the case (i) the first receptor (5) or in the case (ii) one of the first receptors is labelled with visible or coloured particles, particulate direct labellings such as with colloidal gold or latex or polystyrene, the size of which is typically in the range between 5 nm and 100 nm, preferably between 40 nm and 60 nm, or is labelled by means of a further receptor which in case (i) specifically binds the first receptor or in case (ii) binds one of the first receptors, wherein the further receptor is labelled with visible or coloured particles, particulate direct labellings such as with colloidal gold or latex or polystyrene, the size of which is typically in the range between 5 nm and 100 nm, preferably between 40 nm and 60 nm.

12. A method or test according to claim 11 wherein in case (ii) at least one of the first receptors which are not labelled with visible or coloured particles is conjugated with biotin and the second receptor (6) is streptavidin and preferably polystreptavidin so that the first biotinylated receptor or receptors is or are immobilised by way of streptavidin on the test line.

13. A method or test according to one of the preceding claims wherein a control portion such as preferably a control line is provided downstream of the test portion in the transport direction.

14. A method or test according to one of claims 4 to 13 wherein the test strip has an absorption region (4) at its end substantially in the transport direction.

15. A method or test according to one of claims 4 to 14 wherein the test strip is of a width of 3 to 10 mm, preferably about 5 mm, and a length of 50 to 100 mm, preferably 75 mm.

16. A method or test according to one of claims 4 to 15 wherein the length of the conjugate fleece (1) is 5 to 30 mm, preferably about 25 mm, the overlap of the conjugate fleece (1) and the filter in the flow direction is 5 to 15 mm, preferably about 10 mm; when using two conjugate fleeces the length of the first conjugate fleece is preferably about 25 mm, the overlap of the first and second conjugate fleeces in the flow direction is preferably about 12.5 mm, the length of the second conjugate fleece is preferably about 12.5 mm, the overlap of the second conjugate fleece and the filter (2) in the flow direction is preferably about 10 mm, the length of the test or analysis region is 10 to 30 mm, preferably about 20 mm, the width is about 5 mm and the overlap of the test or analysis region and the absorption region in the flow direction is preferably about 1 mm.

17. A method or test according to one of the preceding claims wherein the microorganism is an acid-resistant bacterium, preferably a bacterium of the genus Helicobacter, Campylobacter or the genus Mycobacterium and particularly preferably a bacterium of the species *Helicobacter pylori, Helicobacter hepaticus, Campylobacter jejuni* or *Mycobacterium tuberculosis*, wherein preferably the antigen is the antigen of a catalase, preferably of *H. pylori*.

18. A method or test according to one of the preceding claims wherein the receptor or receptors is or are an antibody or antibodies, a fragment or fragments or derivative or derivatives thereof or an aptamer or aptamers or further preferably a murine antibody or a fragment or derivative thereof or a chimeric, preferably humanized antibody or a fragment or derivative thereof or a binding partner, preferably avidin, streptavidin, polystreptavidin and biotin.

19. A method or test according to one of claims 17 to 18 wherein a mixture of receptors is used for the detection operation, wherein the mixture of receptors functions as a catcher of the antigen if the receptor is used as a detector of the antigen and/or the mixture functions as a detector of the antigen if the receptor is used as a catcher of the antigen and preferably the mixture of receptors is a polyclonal antiserum.

20. A method or test according to one of claims 17 to 19 wherein a mixture of receptors is used for the detection operation, wherein a mixture of receptors functions as a catcher of the antigen and a mixture of receptors functions as a detector of the antigen and preferably at least one mixture is a polyclonal serum.

21. A method or test according to one of claims 17 to 19 wherein a mixture of receptors functions both as a catcher and also a detector of the antigen and preferably the mixture is a polyclonal antiserum.

22. A method or test according to claim 19 wherein the polyclonal antiserum against a lysate of the microorganism was obtained and preferably the lysate is a lysate with enriched antigen and further preferably the lysate is a lysate with depleted immunodominant antigen or wherein the polyclonal antiserum against a purified or a (semi)synthetically produced antigen was obtained and preferably the antigen is an antigen of a catalase.

23. A method or test according to one of claims 17 to 22 wherein the receptor or receptors which functions/function as a catcher of the antigen (a) and/or the receptor or receptors which functions/function as a detector of the antigen (b) is/are respectively replaced by an immune complex which

in case (a) comprises at least one unlabelled antibody which specifically binds the antigen and a labelled antibody which specifically binds said at least one unlabelled antigen, and

in case (b) comprises at least one unlabelled antibody which specifically binds the antigen and an antibody which is immobilised to the test line and which specifically binds said unlabelled antibody.

24. A method or test according to one of claims 19 or 23 wherein the receptor and/or the mixture of receptors binds/bind a confirmation epitope or epitopes.

25. A method or test according to one of claims 18 to 24 wherein the heavy chain of the antibody binding a catalase epitope has at least one of

the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: NYWIH

CDR2: YINPATGSTSYNQDFQD

CDR3: EGYDGFDS

and wherein preferably the DNA sequence coding the heavy chain of the antibody has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: AACTACTGGATTCAC

CDR2: TACATTAATC CTGCCACTGG TTCCACTTCT TACAATCAGG ACTTTCAGGA C

CDR3: GAGGGGTACG ACGGGTTTGA CTCC

and wherein further preferably the light chain of the antibody binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: SASSSVNYMY

CDR2: DTSKLAS

CDR3: QQWSSNPYT

and wherein further preferably the DNA sequence coding the light chain of the antibody has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: AGTGCCAGCT CAAGTGTAAA TTACATGTAC

CDR2: GACACATCCA AATTGGCTTC T

CDR3: CAGCAGTGGA GTAGTAATCC GTACACG

26. A method or test according to one of claims 18 to 25 wherein the heavy chain of the antibody binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: DTYVH

CDR2: KIDPANGKTKYDPIFQA

CDR3: PIYYASSWFAY

and wherein preferably the DNA sequence coding the heavy chain of the antibody has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: GACACCTATGTGCAC

CDR2: AAGATTGATCCTGCGAATGGTAAAACTAAATATGACCCGATA TTCCAGGCC

CDR3: CCCATTTATTACGCTAGTTCCTGGTTTGCTTAC

and wherein further preferably the light chain of the antibody binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: KASQDVGTSVA

CDR2: WTSTRHT

CDR3: QQYSSSPT

and wherein further preferably the DNA sequence coding the light chain of the antibody has at least one of the following CDRs, preferably the CDR3 and more preferably all of the following three CDRs:

CDR1: AAGGCCAGTCAGGATGTGGGTACTTCTGTTGCC

CDR2: TGGACATCCACCCGGCACACT

CDR3: CAGCAATATAGCAGCTCTCCCACG

27. A method or test according to one of claims 18 to 26 wherein the antibodies in the variable regions of the light and heavy chains have the amino acid sequences shown in Figures 1 and 2 or 3 and 4.

28. A method or test according to one of claims 18 to 27 wherein the coding regions of the variable regions of the light and heavy chains have the DNA sequences shown in Figures 1 and 2 or 3 and 4.

29. A method or test according to one of the preceding claims wherein the following steps are carried out with the stool sample prior to

incubation with the antibodies: the stool sample is resuspended in a sample buffer in a ratio of 1:3 to 1:25, preferably about 1:15.

30. A method or test according to one of the preceding claims wherein the same receptor is used for binding to the solid phase as for detection of the epitope.

31. A method or test according to one of the preceding claims wherein the receptor is a monoclonal murine antibody.

32. A method or test according to one of the preceding claims wherein the mammal is a human being.

33. A monoclonal antibody, fragment or derivative thereof which has a V-region which has a combination of the CDRs defined in claims 25 and 26.

34. A monoclonal antibody, fragment or derivative thereof according to claim 33 which has at least one of the V-regions shown in Figures 1 and 2 or 3 and 4.

35. A monoclonal antibody, fragment or derivative thereof according to claim 33 or claim 34 which is a murine antibody or a fragment or derivative thereof or a chimeric, preferably humanised antibody or a fragment or derivative thereof.

36. An aptamer which specifically binds the same epitope as the monoclonal antibody, the fragment or derivative thereof according to one of claims 33 to 35.

37. An epitope which is specifically bound by a monoclonal antibody, fragment or derivative thereof according to one of claims 33 to 35 or the aptamer according to claim 36.

38. An antibody, fragment or derivative thereof which specifically binds an epitope according to claim 37.

39. A kit containing at least one test according to one of claims 2 to 38.

Abstract

The invention concerns a method of detecting an infection of a mammal with an acid-resistant microorganism, which includes the following steps: (a) providing an immunochromatographic test with a sample application region for application of a stool sample of the mammal with an antigen and application of the stool sample, (b) incubating the stool sample with (i) a first receptor under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the receptor; or (ii) at least two different first receptors under conditions permitting a complex formation of the antigen from the acid-resistant microorganism with the at least two first receptors, and wherein the first receptor according to (i) or the first receptors according to (ii) specifically bind(s) an antigen which at least in some of the mammals has a structure after passage through the intestine that corresponds to the native structure or the structure against which a mammal produces antibodies after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide, (c) providing a second receptor immobilised on an analysis region, wherein the second receptor binds an antigen-receptor complex according to (b), and (d) transporting and detecting the formation of at least one antigen-receptor complex according to (b) by accumulation of the antigen-receptor complex at the second receptor in the analysis region. The invention further concerns an immunochromatographic test which is particularly suited to or adapted to carrying out the method according to the invention.